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(54) Title: NUCLEIC ACID CONSTRUCTS AND USES THEREOF FOR DIRECT NUCLEIC ACID INCORPORATION INTO CELLS

(57) Abstract

Disclosed are compositions for incorporating nucleic acid into a cell. Transgene constructs that include a desired nucleic acid sequence of interest may be injected into a cell together with a protein to provide site specific or random incorporation of the nucleic acid sequence into the chromosomal DNA of the cell. The transgene construct and the protein are included in the described compositions together, with the protein facilitating the incorporation of the nucleic acid sequence of interest within the transgene construct, directly into the nucleic acid of a cell in a site-specific manner. In other embodiments, the compositions include a transgene construct and an expression construct, the expression construct including a nucleic acid sequence that encodes an enzyme capable of facilitating the incorporation of the transgene sequence of interest into a cell. The invention further includes methods for incorporating a foreign nucleic acid into a cell employing a microinjection method. In particular embodiments, the method may be employed to introduce foreign nucleic acid into cells that grow in a non-adherent state. In these applications, the cells are immobilized onto a substrate surface that includes an adherent molecule, such as fibronectin. Alternatively, the cells may be stabilized sufficiently to permit microinjection using holding pipettes or through the stimulation of the cells themselves to express molecules on their surface which bind chemical entities included at the surface of a culture plate.

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NUCLEIC ACID CONSTRUCTS AND USES THEREOF FOR DIRECT NUCLEIC ACID INCORPORATION INTO CELLS

This application claims the benefit of U. S. Provisional Application No: 60/033,816, filed 23 December 1996.

TECHNICAL FIELD

The present invention relates to the technical field of nucleic acid incorporation into cells. The invention further relates to the technical field of gene therapy, as techniques described herein may be employed as part of a gene therapy protocol, wherein a number of genetic defects related to particular gene defects may be corrected and/or treated.

BACKGROUND ART

Hematopoietic stem cell gene therapy offers significant promise for treatment of various diseases. At least three requirements must be satisfied for long-term efficacy of stem cell gene therapy: 1) direct genetic modification of hematopoietic stem cells, 2) maintenance of transgene sequences in stem cells and their progeny, and 3) long-term transgene expression in the appropriate cells.

The stem cell compartment, likely to be heterogeneous, consists of rare, long-lived, predominantly quiescent cells that are capable of both long-term reconstitution of the complete hematopoietic system in transplanted hosts and some degree of self-renewal [Ogawa, 1993]. Since stem cells require several months to establish hematopoiesis following transplantation, they must be supplemented with a larger number of short-term reconstituting cells for rapid engraftment [Jones *et al.*, 1995]. For most gene therapy applications, it will likely be sufficient to modify only the stem cells - rapid reconstitution of progenitor-derived unmarked cells would be followed by long term engraftment with genetically marked stem-derived cells.

The introduced therapeutic gene(s) must be successfully transmitted from the stem cell to progeny cells requiring the genetic correction. Since currently available episomal plasmids generally demonstrate only a moderate level of persistence in the absence of selective pressure and artificial human chromosomes await further development [Harrington *et al.*, 1997], chromosomal integration is presently the most viable option for gene maintenance.

Correction of genetic deficiencies will require that the therapeutic gene be expressed in the appropriate cells for the life of the patient. Genetic therapy for the lysosomal storage diseases, irrespective of whether they involve soluble or membrane-bound enzymes, will require life-long production of sufficient levels of the correct enzyme in monocyte/macrophages. Production of insufficient amounts of enzyme will generally lead to redevelopment of disease.

It is well recognized that major problems currently beset the stem cell gene therapy field [Orkin and Motulsky, 1996]. Since the development of retroviral transduction technology in the mid 1980s, this has been a standard gene delivery technique for hematopoietic cells. However, retroviral transduction of primitive hematopoietic cells has revealed significant difficulties in satisfying requirements 1) and 3) identified above. It has been clearly demonstrated in laboratory studies, animal models, and now clinical trials that the presently employed retroviral vectors rarely transduce the quiescent human hematopoietic stem cell, thus failing to fulfill the first requirement [Miller *et al.*, 1990; Kohn *et al.*, 1995; Dick *et al.*, 1996; Dick, 1996; Nolta *et al.*, 1996]. This is evidenced by the extremely low frequency (0.1-1.0%) of gene marked peripheral leukocytes several months after transplant in human trials - indicating stem cell transduction rates of less than or equal to 1% [Kohn *et al.*, 1995; Brenner *et al.*, 1993]. The only early human hematopoietic cells efficiently transduced are cycling progenitors and long term culture initiating cells (LTCICs) [Cassel *et al.*, 1995; Hughes *et al.*, 1989], generally capable of sustaining high levels of blood cell production in vivo only for a finite period (i.e. approximately 2-12 months) - before they are replaced by the progeny of unmodified stem cells. These disappointing results with human stem cells contrast sharply with the efficient retroviral transduction of both stem cells and progenitors routinely demonstrated in the mouse system. Three distinct factors are likely to be responsible for the failure to directly transduce human stem cells: a) the quiescent nature of the stem cells, b) the absence of the appropriate receptors for retroviral envelope on the stem cells [Crooks and Kohn, 1995], and c) that the retroviral transduction conditions, requiring in vitro cycling of stem cells, may actually cause significant loss of stem cell function [Nolta *et al.*, 1996]. Although various techniques are being attempted to overcome these difficulties, the simultaneous solution of all three is not guaranteed. For example, although lentivirus-based vectors may be capable of transducing some non-cycling cells [Naldini *et al.*, 1996], the failure of HIV-1 to infect quiescent primary $CD4^+$ T-lymphocytes

[Zack *et al.*, 1990] (i.e. generating only a partial, labile reverse transcript) suggests that the G_o stem cells may yet prove resistant to transduction. Thus, significant effort is now being devoted to the evaluation of Adeno-associated virus (AAV) vectors for stem cell transduction. However, there is controversy and uncertainty regarding AAV's ability to transduce and stably integrate into quiescent primary cell genomes [Podsakoff *et al.*, 1994; Halbert *et al.*, 1995; Russell *et al.*, 1994].

Even if these gene introduction issues can be solved, significant problems remain in satisfying the third requirement for long-term, cell type-specific expression. For example, the expression of retrovirus-transduced transgenes is frequently silenced in the progeny of transduced human or primate progenitors, and even in progeny of transduced mouse stem/progenitors [Akkina *et al.*, 1994; Challita and Kohn, 1994; Lu *et al.*, 1994]. Furthermore, even in those cells demonstrating some expression, there is significant variability in the level of expression from cell to cell [Sadelain *et al.*, 1995]. As a consequence of this dysregulated expression, hematopoietic cells, although genetically modified with corrective genes, may not efficiently display the corrected phenotype. Interestingly, these same features of dysregulated expression were also observed in the early transgenic mouse expression studies [Kollias and Grosveld, 1992]. Subsequent studies demonstrated that long-term, position-independent, copy number-dependent, cell-type specific expression required not only strong promoter/enhancer elements, but also: a) sufficient genomic sequences to dominantly confer the appropriate chromatin configuration (i.e. an open chromatin conformation in expressing cells) e.g. locus control region (LCR)-like elements [Kollias and Grosveld, 1992; Caterina *et al.*, 1994; Talbot *et al.*, 1990] and perhaps additional elements to shield the integrated sequences from the effects of neighboring chromatin, and b) sufficient intron/exon structure and sequences for high level expression [Brinster *et al.*, 1988]. Unfortunately, the strict packaging requirements for retrovirus vectors (maximum of 8 kb inserted sequences), and the even stricter requirements for AAV vectors (maximum of 4 kb) [Miller *et al.*, 1994], may preclude inclusion of sufficient regulatory sequences and/or intron/exon structure for therapeutic applications requiring even moderately regulated therapeutic gene expression. For example, recent studies examining inclusion of LCR sequences in retroviral and AAV vectors showed extreme variation in the level of transgene expression from cell to cell [Sadelain *et al.*, 1995; Einerhand *et al.*, 1995]. It is also possible that strong splicing signals (i.e. intron/exon

structure) or cryptic splicing signals in transgene sequences will interfere with retroviral packaging of the desired unspliced full-length construct [Einerhand *et al.*, 1995].

The inability of the current technologies to efficiently transduce stem cells is a major deficiency, thus warranting distinct and innovative approaches for transduction.

Electroporation and liposome-mediated transfection technologies have been reported for gene delivery to hematopoietic cells. However, these methods are associated with inherent features that raise questions regarding their appropriateness for stem cell gene therapy. These features include a) the inability of either method to transfect a significant percentage of enriched primary primitive hematopoietic cells of mouse and man [Toneguzzo and Keating, 1986; Harrison *et al.*, 1996], b) significant variation from cell to cell in the copy number of DNA molecules transfected, and c) transient retention of the transgene with only a small minority of cells (1 in 10^4 - 10^5 for electroporation) becoming stably transduced [Philip *et al.*, 1994]. Although less information is available on the recently developed particle-mediated bombardment technique for gene delivery, a recent primary T-cell transfection study reported a 2-10% transfection rate 5 days post bombardment with 1.6 micron gold particle/DNA complexes [Woffendin *et al.*, 1994]. Based on the present inventor's studies with needle sizes in the size range of these particles, stem cell damage would be unacceptably high. It is also not clear that the available non-viral transduction methods would permit co-delivery of proteins with DNA - required for anticipated future applications.

Clinical applications of gene therapy require that therapeutic genes delivered to quiescent stem cells persist both in the self-renewing stem cells and in their maturing/differentiating progeny. Irrespective of the gene transfer method, it is crucial to understand how stem cells, and particularly quiescent stem cells, handle and integrate foreign DNA sequences. Very little is known regarding the fate of DNA (e.g. whether it is integrated into chromosomal DNA) introduced into the nuclei of stem cells, and particularly quiescent stem cells. Viral transduction of primary or quiescent cells may be blocked at several points before integration (e.g. incomplete reverse transcription or transport to the nucleus for retroviruses [Miller *et al.*, 1990; Zack *et al.*, 1990], incomplete conversion of single stranded DNA to double stranded proviral DNA for AAV [Russell *et al.*, 1994]). This has made it very difficult, in the context of the normal AAV or retrovirus life cycle, to focus specifically on the transgene integration process in stem cells. The literature has not revealed any

evidence that integration of microinjected sequences is dependent on cycling. The ability of wild-type HIV-1 to stably transduce quiescent macrophages [Weinberg *et al.*, 1991] and cell-cycle arrested $CD4^+$ HeLa or T lymphoid cells [Lewis *et al.*, 1992] indicates that integration in non-proliferating cells is, in fact, possible. Integration of HIV-1 proviral DNA is mediated by the Integrase enzyme. Some studies indicate that integration of M-MuLV sequences need not occur during mitosis [Roe *et al.*, 1993]; in addition, both chromatin and naked DNA can serve as integration targets in vitro [Pryciak *et al.*, 1992]. Although there is little question regarding the ability of wild-type HIV-1 to integrate in quiescent cells, this has not yet been conclusively demonstrated for HIV-1 based vectors. HIV-1 based vectors have been reported to be capable of establishing a stable transduction intermediate in quiescent Rat 208F fibroblasts [Naldini *et al.*, 1996]. Cells recruited from quiescence even 8 days after original infection had stable transduction frequencies equal to 50% of those infected while in a cycling state.

A need continues to exist in the medical arts for a method that provides for an improved cell viability upon introduction of genetic material into a cell.

DISCLOSURE OF THE INVENTION

The present invention in one aspect relates to the delivery of transgenes, or transgenes complexed with integration enzymes, directly to the nuclei of cells. Such has resulted in the increased incorporation of the transgene into the chromosomal DNA of the cell. In some embodiments, delivery is targeted to primitive cord blood stem cells. This technology will overcome the obstacles to transduction encountered by retroviruses and AAV vectors. The present invention proposes the use of Integrase-mediated integration of appropriately LTR-flanked transgenes in quiescent cells. When considered together with the propensity of retroviral DNAs to integrate into the chromatin of active genes, this indicates to the present inventor that integration is enhanced in the presence of open transcriptionally active chromatin. That is, AAV vectors and Type C retrovirus had not prior to this time been reported to be capable of accomplishing integration readily into quiescent cells.

Generally stated, the present invention in some aspects provides for a method of introducing nucleic acid into the chromosomal nucleic acid of a cell employing a particularly defined composition that includes a protein and a transgene construct. The transgene

construct may be further defined as comprising a nucleic acid sequence of interest flanked by a terminal fragment of a long terminal repeat sequence (LTR), such as a retroviral LTR. The terminal fragment may include a length of about 10 to about 300 nucleotides, or between about 10 to abut 250 nucleotides, or in even further defined embodiments, between about 20 to about 200 nucleotides, in length.

In some embodiments, the protein is defined as an enzyme, such as a retroviral integrase. The protein in other embodiments of the invention may be further defined as a fusion protein, such as an enzyme fused to a nucleic acid binding protein. In yet other embodiments, delivery is particularly targeted to primitive cord blood stem cells. The ability to deliver larger regions of DNA (containing required regulatory elements and significant intron/exon structure) than those packageable in retrovirus or AAV virions will avert the dysregulated expression and silencing frequently observed in the progeny of transduced stem/progenitor cells.

The present invention provides improved compositions containing integrase for delivery of genetic material to cells. In particular embodiments, the cells are hematopoietic stem cells. Microinjection has only rarely been used for primary hematopoietic cells[13]. This has largely been due to the inability to effectively immobilize hematopoietic cells for microinjection, as well as the significant damage caused by standard microinjection needles to the much smaller hematopoietic cells. The present invention reduces both these technical difficulties. In this regard, the invention in one aspect, provides a novel method for temporary immobilization of primitive hematopoietic cells ($CD34^-$, $CD34^+/CD38^-$, $CD34^-/CD38^-/Thy\text{-}1^{lo}$) allowing cells to be rapidly microinjected and then released for subsequent culture and/or transplantation. Fine micropipets with 0.05 - 0.5 micron tip diameter, or 0.12 - 0.2 micron tip outer diameter (O.D.), capable of controllable minimal flow rates, and causing minimal injury to microinjected cells (6-8 micron diameter) are employed in some embodiments of the invention employing the nucleic acid injection preparation described herein. One advantage of the herein described method is that microinjection of minute quantities of either DNA and/or proteins suffice for injection of numerous cells. Until recent years microinjection was not a reasonable option for stem cell modification due to inadequate characterization of the human stem cell phenotype. But with recent identification of enriched populations of primitive human hematopoietic cells likely to include stem cells (e.g. $CD34^+/lin^-/Thy\text{-}1^{lo}$ [14], $CD34^+/CD45Ra^{-/lo}/CD71^{-/lo}/Thy\text{-}1^{lo}$[15], $CD34^+/CD38^-$ [16], $CD34^+/c\text{-}kit^{lo}$[17], $CD34^+/CD38^-/CD33^-/CD19^-/CD45Ra^-/c\text{-}kit^+$[18]), the number

of cells requiring genetic modification has reached a point where gene modification at a single cell level may be a viable alternative.

This innovative approach, focused on modifying one cell at a time as well as multiple cell injection, including the automated injection techniques, provides an economically feasible approach to current cell modification and gene therapy approaches.

Primitive cord blood stem cells will be more efficient than delivery of same with retroviruses and AAV vectors. It is further contemplated that larger regions of DNA, such as those containing required regulatory elements and significant intron/exon structure, than those deliverable by retroviruses or AAV virions will be deliverable by the present microinjection method and will avert the dysregulated expression and silencing frequently observed in the progeny of transduced stem cells.

In some embodiments, the methods include the use of one or more integrases as the particular protein enzyme. By way of example, these integrases include AAV Rep^{78} or retroviral integrases such as M-MuLV, HIV-1, SIV, FeLV, RSV, AMV, or EIAV. These integrases may be in the form of expressed recombinant proteins (expressed either in bacteria, insect cells, or eukaryotic cells) and be either wild-type integrase protein or modified in their amino acid sequence. Modification of the amino acid sequences may occur via several techniques, such as: a) by fusion with additional amino acids to facilitate purification (e.g. histidine tagged, Glutathione S-Transferase (GST) tagged, Maltose Binding Protein (MBP) tagged), b) by fusion with amino acid sequences which provide for binding to specific DNA sequences (DNA binding protein) – and therefore site-preferred or site-specific targeting of integration to specific sites within chromosomal DNA), c) by site specific mutation in the integrase amino acid sequence to provide for greater integrase protein solubility, to provide for increased efficiency in performing the integration reaction, etc.

Although *in vitro* integration can be accomplished at some frequency with ΔLTR_{HIV-1} sequences and $Integrase_{HIV-1}$ alone, inclusion of particular cellular proteins (e.g. the human homolog of yeast transcription factor SNF5 [Kalpana *et al.*, 1994] and DNA bending protein HMG1 [Farnet and Bushman, 1997]) may also be used to even further increase the efficiency of the incorporation and integration. Increased integration efficiency may therefore be achieved by inclusion of such cellular proteins in the DNA/Integrase mixture to be delivered to cells.

The present invention further provides for the co-delivery with the transgene construct and retrovial integrase other viral proteins that are normally present in the retroviral pre-integration complex. For example, M-MuLV gag proteins are present in the M-MuLV pre-integration complex, potentially increasing the efficiency of the integration reaction.

BRIEF DESCRIPTION OF DRAWINGS

The following drawings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these drawings in combination with the detailed description of specific embodiments presented herein.

FIG. 1 Introduction of transgenes into cells together with specific enzymes designed to facilitate integration. Constructs flanked by the termini of retroviral long terminal repeats (LTR) will be co-delivered with retroviral integrase. This approach could apply to any retrovirus (e.g. a lenti-virus such as Human Immunodeficiency Virus type 1 or a type C retrovirus such as Moloney Murine Leukemia Virus). Constructs flanked by the AAV inverted terminal repeats (ITR) will be co-delivered with AAV Rep^{78}. Although this schematic shows introduction of both the transgene construct and the integration enzyme by microinjection, both transgene and enzyme could potentially be delivered by other means, such as electroporation, liposome-mediated gene transfer, or particle-mediated bombardment. In addition, it is possible that the integration enzyme would not need be delivered as protein. Rather, it could be co-delivered as a DNA expression construct together with the transgene targeted for integration. If only the transgene, and not the integration enzyme expression construct, was flanked with LTR or ITR sequences, this would facilitate integration of only the desired transgene.

FIG. 2. Strategies for integration. Simple introduction of the transgene and regulatory elements into the cell, when resulting in integration, should result in integration at random sites, with possible head-to-tail concatamers. Simple addition of the AAV ITR sequences are expected to provide increased rates of integration. High frequencies of integration have been observed for rep$^-$ AAV vectors, which like this construct, only contain ITR sequences and no Rep^{78}. The precise structure of the rep$^-$ AAV DNA prior to integration may either exist as a linear duplex flanked by ITRs, or be circularized in the nucleus prior to integration. Hence, the present invention proposes that increased rates of integration may

result from circularization of the 2 ITR-containing construct. In the absence of the rep gene products the integrants generally show no specificity for the AAVS 1 region of Chromosome 19. Addition of Rep^{78} protein (shown in the next construct) is expected to increase the preference of integration for Chromosome 19, since Rep^{78} recognizes DNA sequences both in the AAV ITR and the Chromosome 19 AAVSI region. In the next construct, the gene construct is flanked by sequences derived from a retroviral LTR. For example, the only M-MuLV LTR sequences required for *in vitro* integration reactions are the extreme 10-12bp LTR termini. The M-MuLV viral enzyme Integrase interacts with these sequences to facilitate integration *in vitro* and *in vivo*. There appears to be no site specificity for integration of M-MuLV based vectors, although there is some preference with respect to nucleosomal conformation. Shown in the next part of the diagram is the interaction with the LTR sequences of a fusion protein between Integrase and a DNA binding protein. This DNA binding domain could either be derived from an existing DNA binding protein, or a novel protein could be designed and synthesized to have a high affinity for specific genomic DNA sequences. For example, it is possible that specific targeting of transgenes to the globin locus would facilitate their expression specifically in erythroid cells.

FIG. 3. Diagramed are the two fusion proteins expressed and purified in our laboratory to facilitate integration of microinjected DNA sequences. Shown above is a fusion between the maltose binding protein and the Rep^{78} protein of AAV. This was expressed from an MBP-Rep^{78} expression construct (Batchu *et al.*, 1995). The MBP-Rep^{78} fusion protein, purified in the identical manner, was reported to exhibit *in vitro* binding to the AAV ITRs, endonuclease activity, and helicase activity. Should the MBP sequences interfere with integration activity (in the cell), they may be removed from Rep^{78} by cleavage with factor Xa (a factor Xa cleavage site is present at the site of fusion between MBP and Rep^{78}). Shown below is a similar fusion between MBP and the integrase gene of M-MuLV. The M-MuLV integrase gene was cloned from a construct described in Jonsson *et al.*, (1993) – and cloned into the pMALc2 vector used for expression of MBP-Rep^{78}. However, the strategy described is not limited to MBP-fusion proteins in the practice of the present invention. It could equally well employ any expression and purification strategy – for example, GST-fusions, histidine-tagged expression and purification, baculovirus expression vs. *E. coli* expression, and expression in eukaryotic cells.

FIG. 4A and FIG. 4B. Purification of MBP-Rep^{78} fusion protein. FIG. 4A: bacterial lysates from pMAL-Rep^{78} transformed bacteria – grown either in the absence of IPTG, or

presence of IPTG (to induce MBP-Rep^{78} expression). FIG. 4B: A new band of approximately 120 kD is present in the +IPTG lane. Bacteria grown in the presence of IPTG were sonicated, and the supernatant passed twice over an amylose column. MBP-Rep^{78} was eluted from the column by addition of maltose. There is an additional co-purifying band, running at a somewhat lower molecular weight. [Batchu *et al.*, 1995] postulated that this may be a product of a favored proteolytic cleavage of the full length MBP-Rep^{78} or due to a favored early termination product. By scanning the gel, the purity of this preparation is estimated to be 90-95%.

FIG. 5. Purification of MBP-Integrase fusion protein. An expression construct fusing the MBP sequences with the M-MuLV Integrase gene [Johnson *et al.*, 1993] was prepared. Sequencing of the expression construct confirmed the appropriate in-frame fusion. The MBP-Integrase protein expressed and purified similarly to that described in FIG. 4 above for MBP-Rep^{78}. Bacteria grown in the presence of IPTG were sonicated, and the supernatant passed once over an amylose column. A strong band running slightly slower than the 85 kD molecular weight marker is consistent with the estimated 88 kD fusion protein. The purity of this preparation is estimated to be in the range of 70-80%.

FIG 6. Sample constructs to be employed in assaying integration enzyme-mediated integration; prsGFP-MGMT contains both the humanized red shifted Green Fluorescent Protein gene (GFP; from Gibco BRL, pGREEN) and the human O^6-methylguanine DNA methyltransferase gene. All transgenes in this and the following constructs are driven by the phosphoglycerate kinase (pgk) promoter sequences. This particular construct is flanked by rare enzyme sites (Fse 1, Pac 1, Asc 1, Sfi 1) to readily permit removal of bacterial plasmid sequences to obtain linearized transgene sequences only (by restriction enzyme digestion and, if necessary, eluting the correctly sized fragment from a preparative agarose gel). prsGFP-MGMT/ITR contains the AAV2 ITR sequences flanking the prsGFP-MGMT sequences. Again, linear sequences absent of bacterial plasmid sequences will be obtained by digestion with Fse I and Sfi I enzymes. It is also possible to obtain this construct in a circularized form by converting the Sfi I site to an Fse I site, digesting with Fse 1, and religating to form a 2-ITR containing circle. Since this may be the appropriate substrate for AAV integration, this circularized construct may integrate more easily than the linearized, either in the absence or presence of Rep^{78}. The last construct consists of linearized rsGFP-MGMT/ΔLTR obtained by PCR amplification with primers containing, at their 5' ends, 20 bases of sequence corresponding to HIV-1 LTR termini. PCR amplification was used to generate double

stranded linear constructs flanked by the appropriate HIV-1 termini. If the 20 bp of sequence are not sufficient for efficient integration, the strategy may be modified to include additional LTR sequences.

FIG 7. Generation of pgk-tk/ΔLTR_{HIV-1}. Shown is the construct containing the herpesvirus type 1 thymidine kinase (tk) gene, together with the phosphoglycerate kinase (pgk) promoter. Also shown are SV40 splice donor/acceptor and polyadenylation sequences. PCR primers were designed to generate a 2.74 kbp linear fragment containing the pgk and tk sequences, spanned by the terminal sequences of the HIV-1 LTR. The primer (HIVU3; 36 bases) employed upstream of the Fse I site included 20 bases corresponding to the terminus of the U3 region (underlined) and 16 bases corresponding to sequences in the original pCMVβ vector (5' ACTGGAAGGGCTAATTCACTGTTGGGAAGGGCGATC 3') (SEQ ID NO: 1). The primer (HIVU5; 35 bases) employed downstream of the Sfi site included 20 bases corresponding to the terminus of the U5 region (underlined) and 15 bases corresponding to sequences in pCMVβ (5' ACTGCTAGAGATTTTCCACACAGGAAACAGCTATG 3') (SEQ ID NO: 2). Amplified linear double stranded DNA was obtained after 30 cycles of PCR amplification employing Vent DNA polymerase (New England Biolabs). After PCR, the PCR product was extracted with chloroform, precipitated with sodium acetate and alcohol, resuspended in TE, subjected to electrophoresis in a 1% agarose gel, gel purified using a Geneclean kit (Bio 101), precipitated with sodium acetate and alcohol, resuspended in water, filtered through a 0.1 micron filter, and stored at –20 deg C until use.

FIG 8. pgk-tk/ITR. Shown is the pgk-tk construct, flanked by AAV ITRs to generate pgk-tk/ITR. Linearized ITR-flanked pgk-tk sequences are obtained by digestion with Fse I and Sfi I, and then gel purified to eliminate bacterial plasmid sequences, since bacterial plasmid sequences have been shown to interfere with gene expression in transgenic mice.

FIG 9. Generation of covalently closed circles for facilitating integration. cir-rsGFP-MGMT/ITR will be employed as a circular double-stranded DNA in some aspects of the present invention. A plasmid backbone containing the AAV ITRs has been constructed, and the rsGFP-MGMT sequences inserted between the Pac I and Asc I sites to generate prsGFP-MGMT/ITR. Linearized ITR-flanked rsGFP-MGMT sequences have been obtained as described above by digestion with Fse I and Sfi I. Circularized rsGFP-MGMT/ITR sequences, absent the plasmid backbone, will be generated as diagramed in Fig. 9. The Sfi

I site was converted in prsGFP-MGMT/ITR to create a second Fse I site. This will be linearized and the backbone plasmid sequences removed via Fse I digestion, religate to circularize at the Fse I site, and purified for circular molecules on an agarose gel prior to microinjection.

BEST MODES FOR CARRYING OUT THE INVENTION

The present invention in some embodiments provides a method for the transduction of hematopoietic stem cells (HSCs) and thus an alternative strategy for their direct genetic modification by: 1) direct delivery of DNA sequences into the nuclei of HSCs by microinjection; 2) integration of microinjected transgene sequences in the chromatin of HSCs and persistence of those sequences in the progeny of said HSCs; and 3) microinjection of sufficiently large (15-25 Kb) transgenic DNA constructs containing regulatory elements, such as promoters, enhancers and LCRs, and intron/exon structure necessary for appropriate long-term, cell type-specific expression of the introduced transgenes; and 4) microinjection of DNA/protein mixtures with the protein(s) included in the injection sample having gene targeting activities.

Genetically modified HSCs prepared according to the methods of the present invention can be employed for gene therapy applications once said modified HSCs have been delivered to humans for long-term reconstitution.

According to other embodiments of the present invention, hematopoietic stem cells that have been modified by microinjection of foreign material can be used to treat a variety of physiological disorders such as, by way of example and without limitation, AIDS, thalassemia, sickle cell anemia, and adenosine deaminase deficiency.

The physiological disorders contemplated within the invention will be responsive to gene therapy. By "responsive to gene therapy" is meant that a patient suffering from such disorder will enjoy a therapeutic or clinical benefit such as improved symptomatology or prognosis.

As indicated above, one aspect of the present invention relates to the use of modified HSCs. as cellular vehicles for gene transfer. The genes, or transgenes, can be any gene having clinical usefulness, for example, therapeutic or marker genes or genes correcting gene defects (e.g. mutant hemoglobin genes in thalassemia or sickle cell anemia) in blood cells. In some embodiments, the primary human cells are blood cells. The term "blood cells" as

used herein is meant to include all forms of blood cells as well as progenitors and precursors thereof, as hereinabove described.

Thus, in one embodiment, the invention is directed to a method of enhancing the therapeutic effects of HSCs, comprising: (i) microinjecting into the HSCs of a patient a DNA segment encoding a product that enhances the therapeutic effects of the human primary cells; and (ii) introducing the genetically modified HSCs resulting from step (i) into the patient.

The DNA produces the agent in the patient's body, and, in accordance with such embodiment, the agent is expressed at the tissue site itself. Similarly, as hereinabove indicated, HSCs which are genetically engineered need not be targeted to a specific site and, in accordance with the invention, such engineered HSCs and their progeny function as a systemic therapeutic; e.g., a desired therapeutic agent can be expressed and secreted from the cells systemically.

More specifically, there is provided a method of enhancing the therapeutic effects of HSCs. that are infused in a patient, comprising: (i) microinjecting into the HSCs of a patient a DNA segment encoding a product that enhances the therapeutic effects of the blood cells; and (ii) introducing cells resulting from step (i) into the patient.

The primary human blood cells which are the progeny of modified HSCs and which can be used in the present invention include, by way of example, leukocytes, granulocytes, monocytes, macrophages, lymphocytes, and erythroblasts. For example, stem cells from thalassemic or sickle cell anemia clients that are genetically modified with the appropriate hemoglobin gene may give rise to genetically corrected red blood cells.

The DNA carried by the HSCs can be any DNA having clinical usefulness, for example, any DNA that directly or indirectly enhances the therapeutic effects of the cells. Alternatively, the DNA carried by the HSCs can be any DNA that allows the HSCs to exert a therapeutic effect that the HSCs would not normally exert. Examples of suitable DNA, which can be used for genetically engineering, for example, blood cells, include those that encode cytokines such as tumor necrosis factor (TNF), interleukins (for example, interleukins 1-12), globin genes, DNA-repair genes, drug-resistance genes and HIV (Human Immunodeficiency Virus) resistance genes.

The DNA which is used for transducing the human cells can be one whose expression product is secreted from the cells. Alternatively, it may encode for gene products retained within the cell. The human cells can also be genetically engineered with DNA which functions as a marker, as hereinafter described in more detail.

In one aspect, the inserted genes are marker genes which permit determination of the traffic and survival of the transformed cells *in vivo*. Examples of such marker genes include the neomycin resistance (neoR) gene, multi-drug resistant gene, thymidine kinase gene, β-galactosidase, dihydrofolate reductase (DHFR) and chloramphenicol acetyl transferase.

The HSCs are genetically engineered *in vitro*. For example, cells may be removed from a patient and stem cells isolated; genetically engineered *in vitro* with DNA encoding the therapeutic agent, with such genetically engineered HSCs being readministered along with a pharmaceutically acceptable carrier to the patient. Such a treating procedure is sometimes referred to as an *ex vivo* treatment.

As part of a clinical treatment regiment for a patient having cancer or at risk of developing cancer, the progeny of the modified HSCs provide a population of primary human cells that express the product of the genetic foreign material microinjected into the parent HSCs.

The pharmaceutically acceptable carrier may be a liquid carrier (for example, a saline solution) or a solid carrier; e.g., an implant. In employing a liquid carrier, the engineered cells may be introduced parenterally, e.g., intravenously, sub-cutaneously, intramuscularly, intraperitoneally, or intralesionaly.

The following abbreviations have been used in the preparation of this disclosure.

pCMV-β	DNA plasmid expressing the β-gal reporter gene under control of the cytomegalovirus (CMV) promoter/enhancer sequences.
FITC-Dextran	FITC coupled to dextran
CD34+CD38-Thy-1+	the $CD38^{-}Thy\text{-}1^{+}$ (actually $Thy\text{-}1^{lo}$) subpopulation of $CD34^{+}$ cells isolated by FACS.
IMDM	Iscove's Modified Dulbeccos Media.

The foregoing will be better understood with reference to the following examples which detail certain procedures according to the present invention. All references made to these examples are for the purposes of illustration. They are not to be considered limiting as to the scope and nature of the present invention.

EXAMPLE 1
DELIVERY OF FITC-DEXTRAN TO IMMOBILIZED CD34$^+$ CELLS

Purification and Culturing of CD34$^+$ CELLS

The CD34$^+$ antigen, present on approximately 0.5-1.0% of mononuclear bone marrow and umbilical cord blood cells, marks measurable human hematopoietic stem and progenitor cells. Umbilical cord blood cells were obtained from normal human fetal deliveries, and mononuclear cells were purified by centrifugation over Ficoll-hypaque. CD34$^+$ cells were isolated by immunomagnetic selection with the Miltenyi MiniMACS CD34 Multisort Isolation Kit (involves (1) incubation of cells with anti-CD34 antibody coupled via dextran to immunomagnetic particles, (2) isolation of magnetically-labeled cells by passing through a column attached to a magnet, (3) release of cells from magnetic particles by cleavages with dextranase, (4) separation of cells from magnetic particles by passing through column attached to a magnet). Subsequent FACS analysis, with another anti-CD34 antibody recognizing a different CD34 epitope, demonstrated that the cells were 90% pure for CD34 expressing cells. Purified cells were maintained overnight (18 hrs) in serum free medium (Iscoves Modified Dulbecco's Medium (IMDM, Gibco) supplemented with bovine serum albumin (2%, StemCell Technology), insulin (10 micrograms/ml,), transferrin (200 microgram/ml, ICN), 2-mercaptoethanol (0.05 mM, Sigma), low-density lipoprotein (40 microgram/ml, Sigma), and pen-strep (100 units and 50 microgram/ml, respectively) containing 20 ng/ml human Flt-3 ligand (Peprotech), 20 ng/ml human Interleukin-3 (IL-3, Peprotech), and 20 ng/ml human Stem Cell Factor (SCF, Peprotech) [IMDM/F-3-S] at 37° C with 5% CO_2.

Preparation of Fibronectin-Coated Surface

A 6 mm glass cloning ring was attached via vaseline to a 35 mm tissue culture dish (Corning). The dish surface enclosed by the cloning ring was coated with fibronectin by adding 30-50 microliters of a 50 microgram/ml fibronectin solution (Boehringer Mannheim, #1051-407) in phosphate buffered saline (PBS, Sigma), and incubating overnight at 4 °C (alternatively, can be for 45 min. at room temperature). Excess fibronectin-containing solution was removed from the cloning ring immediately prior to addition of cells.

Attachment of $CD34^+$ Cells to Fibronectin-Coated Dish

After overnight culture, cells were prepared at a concentration of 8×10^4 cells/ml in IMDM/F-3-S. This cell-containing media (25 microliters containing approximately 2000 cells) was mixed with 25 microliters of media (IMDM) conditioned 2 days by the TS2/16.2.1 hybridoma cell line (ATCC #HB-243 which produces antibody reactive with Integrin beta$_1$ -human CD29). The 50 microliters of cell/antibody mixture was placed into a 6 mm glass cloning ring enclosing the fibronectin-coated surface. Cells, in the presence of antibody, were allowed to attach to fibronectin for greater than 30 min. at 37 °C in the presence of 5% CO_2. Subsequently, 1 ml of IMDM/F-3-S was added outside the cloning ring, and the 35 mm plate containing cells and cloning ring was spun at 600 rpm for 5 min (Beckman low-speed GS-6R centrifuge, swinging bucket rotor, brake off).

Microinjection of $CD34^+$ Cells with FITC-Dextran

Fine glass microinjection needles were prepared from thin-walled borosilicate glass capillaries (Sutter, 1.2 mm O.D., 0.94 mm I.D.) with an automated pipet puller (Sutter, P-87, 3 mm box filament). Scanning Electron Transmission (SE) microscopy was used to determine the outer diameter of microinjection needles pulled with the identical program; O.D.s between 0.17 and .22 micron were obtained. FITC-dextran (150,000 M.W., Sigma) at a concentration of 0.25% (weight per volume) in 50 mM Hepes (pH 7.2/100 mM KCl/5 mM NaH_2PO_4) was passed through a 0.02 micron filter (World Precision Instruments) and centrifuged at high speed (10,000 rpm, IEC Centra-4b) before loading via an Eppendorf microloader into microinjection needles. Microinjections were performed manually with a Narishige micromanipulator mounted on an Olympus OMT-2 inverted microscope (with heated stage) with a 40x phase dry objective. Pressure for fluid delivery was provided by an SAS 10/2 Screw Actuated Air micro-injection/aspiration syringe. The cloning ring was removed immediately before microinjection. After flow of fluid from the needle was confirmed, 60 cells (in approximately 30-45 minutes) were microinjected with approximately 2-10 femtoliters of FITC-dextran. Although delivery of material was targeted for the nucleus, some material was delivered to the cytoplasm.

Monitoring and Subsequent Culture of Microinjected Cells

After microinjection, a larger cloning ring (8 mm diameter) was placed around the region of microinjected cells. Delivery of FITC-dextran to cells was monitored by

fluorescence microscopy with a Nikon Diaphot 300 inverted microscope with fluorescence attachment (FITC.). Both bright-field and fluorescence images can be captured and saved with a Hamamatsu chilled CCD camera and controller, Sony Trinitron monitor, frame grabber, and networked Gateway 486-25. Within 30 min. after microinjection, approximately 35 FITC-positive cells (out of 60 total; 58-67%) were clearly identifiable. A significant number of the cells clearly showed nuclear localization of fluorescence; some showed both nuclear and cytoplasmic localization, and a small number showed only cytoplasmic fluorescence. Twenty-four hours post microinjection, 19 fluorescent cells (32%) were still visible; seventy-two hours post microinjection, 16 fluorescent cells (32%) were visible.

EXAMPLE 2
TRANSGENE CONSTRUCT

The present example is provided to demonstrate the utility of the present invention for providing transgene constructs that include a nucleic acid sequence encoding a gene of interest, such as a therapeutic gene, and the successful stable incorporation and expression of the therapeutic gene, in a cell. The gene may be any mammalian gene, or pharmacologically active fragment thereof, and particularly a human gene.

The present example describes a number of transgene constructs that express both the red shifted Green Fluorescent Protein (rsGFP) reporter gene and the human O^6-methylguanine DNA methyltransferase (MGMT) gene. The MGMT gene is one that would provide chemotherapeutic agent resistance to cells, and hence would provide a desired and useful treatment for a patient exposed to chemotherapeutic agents. For example, such may include patients with cancer/tumors undergoing chemotherapy as part of an anti-cancer regimen.

Since the rsGFP reporter permits rapid and sensitive assessment of expression, it will permit a rapid determination of nuclear-specific gene deliver and transgene persistence. The human MGMT gene was chosen because MGMT transgene expression in mouse stem cells is sufficient to protect them from the toxic effects of alkylating agents such as BCNU. The present invention provides methods for targeted expression of MGMT in human stem cells, and further demonstrates the utility of the invention for *in vivo* enrichment of transduced stem cells.

In some embodiments, the invention provides a method for the stable transduction of primitive cord blood cells, and the preparation of immature cord blood populations enriched in SRC activity. Improved methods for stable integration frequency in cell co-injected with constructs flanked by HIV-1 LTR or AAV ITR sequences together with HIV-1 Integrase or AAV Rep^{78} are also provided.

Four particular transgene constructs are defined in FIG. 6. All include MGMT.

EXAMPLE 3
HUMAN GENE THERAPY METHODS

The present example demonstrates the utility of the present invention for providing a gene therapy treatment for humans.

The number of stem cells that need be delivered to humans for long term reconstitution may be extrapolated from mouse, large animal, and human studies to permit a reasonable estimate. Since the genetic therapies under consideration will frequently be directed to children, these estimates are based on their smaller body weight. Although a significant number of unmarked, short-term reconstituting cells may be co-delivered to better facilitate rapid engraftment and survival, the focus here is on the much smaller number of gene-modified, long-term reconstituting stem cells. a) Three independent mouse studies have reported long-term reconstitution with as few as 20 marrow cells [Halbert *et al.*, 1995], 10 marrow cells [Russell *et al.*, 1994], or even 1 marrow cell (20% of mice reconstituting [Akkina *et al.*, 1994]). If direct scaling by weight alone is appropriate, an average reconstitution requirement of 5 cells for mice would extrapolate into approximately a 5,000 marrow cell requirement for a human child. b) In human marrow transplantation, the minimal dose typically delivered is 1×10^8 nucleated cells per kg body weight [Challita and Kohn, 1994], equivalent to 2.5×10^9 cells for a 25 kg child. Experimental data and modeling of feline hematopoiesis indicate that the stem cell frequency is approximately 1 in 1.7×10^6 marrow cells [Lu *et al.*, 1994]. If this same frequency holds for human marrow, delivery of 2.5×10^9 cells corresponds to delivery of 1450 stem cells. c) Children reconstitute with as little as 30 mls of transplanted cord blood, likely due to the significant proliferative potential of primitive hematopoietic cord blood cells [Sadelain *et al.*, 1995]. Assuming approximately $1.5 - 3 \times 10^8$ nucleated cells in this volume, with a stem cell frequency of 1 in 10^5 to 10^6 this translates into successful engraftment with as few as 150 -3,000 stem cells. d) Evidence from engraftment of human cord blood cells in NOD/SCID mice suggests a very close relationship

(if not equivalence) between NOD/SCID reconstituting cells (SRCs) and human stem cells. SRCs are present at a frequency of ~ 1 in 10^4 $CD34^+$ cells [Kollias and Grosveld, 1992; Caterina *et al.*, 1994]. Thus, 30 mls of cord blood (with approximately 3×10^7 mononuclear cells, 1% of which are $CD34^+$) contain approximately 30 SRCs. Even if the seeding efficiency of SRCs in NOD/SCID mice is only 10%, this translates into 300 SRCs. Thus, all four calculations suggest a number of stem cells in the range of about 150 to about 5000 would be useful as a treatment in the practice of the present invention. Successful reconstitution is affected by stem cell delivery and engraftment by short-term progenitors. It is possible therefore that the stem cell requirements are even less than those calculated in b) and c) above.

Although the present hematopoietic stem cell-oriented work has been with manual microinjection (100-200 injected cells/hr), the presently disclosed methods are expected to easily accommodate the higher-speed (300-600 cells/hr) automated injection. The automated microinjection system may well be adequate for clinical gene therapy applications. Computer automated systems, capable of 1500 cell injections per hour [Pepperkok *et al.*, 1988], may be employed to microinject a sufficient number of stem cells for transplantation (i.e. 1000-10,000 cells depending on the stable transduction frequency). Any significant *in vitro* or *in vivo* expansion of stem cells [Emerson, 1996], as well as together with selection for marked cells, would further decrease the number of microinjected stem cells required for engraftment.

EXAMPLE 4
DNA CONSTRUCTS AND GENE THERAPY

A significant fraction of corrected stem cells present *in vivo* may be accomplished by *in vitro* selection for marked stem cells prior to engraftment (so that only the successfully transduced cells are transplanted into the patient) and/or by subsequent *in vivo* selection for marked stem cells (to enrich for gene marked stem cells at the expense of endogenous, unmarked stem cells). This will generally require transduction of stem cells with two independently regulated genes present on the same DNA construct: the selectable gene targeted for expression in stem/progenitor cells and the therapeutic gene (e.g. ADA or globin) - targeted for expression in the required cell type. Genes potentially useful in in vitro selection of transduced stem cells include rsGFP or truncated nerve growth factor receptor

(tNGF-R). Transduction of stem cells with the human O^6-methylguanine DNA methyltransferase (MGMT) gene will enable *in vivo* selection of surviving, marked stem cells by briefly treating patients with alkylating agents of the nitrosourea class (e.g. 1,3-bis (2-chloroethyl)-1-nitrosourea; BCNU).. Whereas most anti-neoplastic drugs (e.g. Taxol) are toxic to cycling hematopoietic progenitors, sparing the quiescent hematopoietic stem cells, nitrosoureas, such as BCNU, also exert their DNA-damaging and toxic effects directly on the stem cells. MGMT, which removes O^6-alkylguanine induced in DNA by various alkylating agents, is normally expressed at very low levels in hematopoietic stem/progenitor cells. However, when exogenously expressed in cells, MGMT confers cell resistance to BCNU, CCNU, dacarbazine, N-methyl-N'-nitro-N-nitrosoguanidine, temozolomide, and streptozotocin. For example, mice expressing MGMT in their stem cells were resistant to BCNU-induced hematosuppression [Maze *et al.*, 1996]. The human multiple drug resistance gene (MDR-1) has been proposed for *in vivo* selection of transduced stem cells. Human stem cells already constitutively express MDR-1 [Chudhary and Roninson, 1991]. Hence, the enrichment for transduced cells by MDR-1 resistant drugs (e.g. taxol) as employed in the practice of the present invention is expected to occur at the level of progenitors but not stem cells. As is true for any proposed *in vivo* selection (e.g. BCNU, taxol) for marked hematopoietic cells, drug toxicity for other organs and cells will be minimized. MGMT transgene expression, by itself, is expected to confer resistance in hematopoietic cells to agents such as BCNU employed in high-dose or repetitive chemotherapy for breast and other cancers [Maze *et al.*, 1996].

In one aspect, gene therapeutic applications of stem cell microinjection may include the following elements: Approximately 1-10 x 10^3 highly enriched stem cells will be obtained from cord blood, and will be temporarily immobilized. Microinjection of these cells will deliver a reproducible volume— containing DNA, and in some embodiments, also include integration enzyme(s)—such that 1-3 copies of the DNA are successfully integrated per cell. Microinjected DNAs of 15-25 kb in size, containing two independently regulated transgenes, will be integrated without rearrangement. One transgene, targeted for expression in stem cells, will provide for *in vitro* (e.g. rsGFP, or truncated nerve growth factor receptor; tNGF-R) or *in vivo* (e.g. MGMT) selection of transduced stem cells. The therapeutic transgene (e.g. ADA for ADA SCID, globin for hemoglobinopathies, MDR-1 for chemoresistance) will be targeted for expression in the appropriate hematopoietic cells. Microinjection would also be an appropriate method for eventual nuclear delivery of artificial human chromosomes and/or

episomal plasmids capable of persistent maintenance. In some embodiments, cell viabilities greater than 80% post microinjection and stable transduction frequencies greater than 25% are expected to be provided.

EXAMPLE 5
PURIFICATION AND BIOLOGICAL ASSAYS OF PRIMITIVE CORD BLOOD CELLS

$CD34^+$, $CD34^+/CD38^-$, and $CD34^+/CD38^-/Thy\text{-}1^{lo}$ populations of cord blood cells are provided. $CD34^+$ hematopoietic cells, representing approximately 0.5 - 1.0% of nucleated umbilical cord blood and bone marrow cells, comprise all measurable human stem/progenitor activity. The $CD34^+/CD38^-$ phenotype (approximately 5-10% of $CD34^+$ cord blood cells) characterizes an even more primitive subpopulation. Cells capable of engrafting human hematopoiesis in the immunodeficient NOD/SCID mice—considered to be more primitive than LTCICs and colony forming cells—are present exclusively in the $CD34^+/CD38^-$ population [Dick *et al.*, 1996; Dick, 1996]. $CD34^+/CD38^-$ cord blood cells are highly enriched in the primitive $CD34^+/CD45Ra^{-/lo}/CD71^{-/lo}$ phenotype identified by Mayani *et al.* [Mayani *et al.*, 1993]. Their further demonstration—that the $Thy\text{-}1^{lo}$ subset of $CD34^+/CD45Ra^{-/lo}/CD71^{-/lo}$ cells represents an even more primitive population of cells—is consistent with the characterization of human fetal liver stem cells as being $CD34^+/lin^-/Thy\text{-}1^{lo}$. The present inventors have observed that $CD34^+/CD38^-/Thy\text{-}1^{lo}$ cells (approximately 10-25% of $CD34^+/CD38^-$ cells) are significantly more enriched than $CD34^+/CD38^-/Thy\text{-}1^-$ cells in LTCICs that produce colony forming cells generating large numbers of progeny. Based on the above considerations, $CD34^+$, $CD34^+/CD38^-$, and $CD34^+/CD38^-/Thy\text{-}1^{lo}$ phenotypes represent increasingly enriched populations of human stem cells.

Protocols to purify and functionally assay these particular cell populations—to permit the microinjection and stable transduction assays—are thus to also be provided.

EXAMPLE 6
PURIFICATION OF $CD34^+/CD38^-$; AND $CD34^+/CD38/THY\text{-}1^{lo}$ CELLS

Umbilical cord blood cells were obtained on a weekly basis (2-4 samples per week; total of 100-250 mls) from normal deliveries at UTMB, and pooled mononuclear cells isolated by centrifugation over Ficoll-hypaque. $CD34^+$ cells are isolated by immunomagne

selection with the Miltenyi MiniMACS CD34 Multisort Isolation Kit. Isolated cells are obtained free of attached magnetized particles (by cleaving the dextran antibody-particle linker with dextranase). Recovery was typically 70-90% of the expected CD34 cell number (100 mls of cord blood typically yields 1-2 x 10^8 mononuclear cells, with CD34 isolation giving 1-2 x 10^6 cells with 90-95% viability and 80-95% CD34 positivity). Fluorescence activated cell sorting (FACS) with a Becton-Dickinson FACS Vantage instrument is then employed to isolate specific $CD34^+$ cell subpopulations. Cells satisfying the sort criteria are either bulk sorted into tubes or a pre-specified number are deposited into individual wells of a 96 well plate via an Automated cell deposition unit (ACDU). For purification of $CD34^+/CD38^-$ cells, the enriched $CD34^+$ cells are stained with PerCP-CD34 and PE-CD38 antibodies, and cells satisfying both the $CD34^+/CD38^-$ and low side and forward scatter criteria are isolated. $CD34^+/CD38^-$ cells typically comprise approximately 5-10% of the total $CD34^+$ cells; with an actual sort recovery rate of 50%, we yield approximately 2 - 5 x 10^4 $CD34^+/CD38^-$ cells per sort. Excellent purity of the sorted $CD34^+/CD38^-$ cells was obtained. The primitive nature of the sorted $CD34^+/CD38^-$ cells was substantiated by the vast majority of them exhibiting the $CD45Ra^{-/lo}/CD71^{-/lo}$ phenotype. The $CD34^+/CD38^-$ population may in some instances subdivide into Thy-1^{lo} (approximately 10-25% of the $CD34^+/CD38^-$cells) and Thy-1^- (approximately 75-90% of $CD34^+/CD38^-$ cells) subsets. Approximately 2-10 x 10^3 $CD34^+/CD38^-$/Thy-1^{lo} enriched cells can be typically recovered in such sorts. Such provides a method for enhancing a population of cells for primitive ($CD34^+/CD38^-$, $CD34^+/CD38^-$/Thy-1^{lo}) populations of cord blood cells to be used in the microinjection and functional assays.

EXAMPLE 7
EXPRESSION OF rsGFP BY MICROINJECTED $CD34^+$, $CD34^+/CD38$/THY-1^{lo} CELLS

Transient reporter gene expression in cells microinjected with DNA is demonstrated in the present example. The rsGFP reporter is the represented DNA employed. It permits rapid assessment of gene expression, without the need for additional antibody labeling or enzymatic assays.

Cells are isolated and attached to fibronectin. Cells are generally microinjected with about 100 to about 250 ng/microliter solution, in microinjection buffer, of plasmid DNA expressing the humanized rsGFP protein under control of the cytomegalovirus (CMV)

promoter/enhancer. Microinjection needles of 0.2 +/- 0.02 micron O.D. are presently employed. rsGFP expression is monitored by fluorescence microscopy using a filter set optimized for rsGFP detection. rsGFP expression in 10-15% of injected cells, 5-24 hours post injection was generally observed. Although this frequency gradually decreases with increasing time of culture and observation, expressing cells (and a smaller number of dividing, expressing cells) were observed as late as 4-5 days after microinjection. Since the FITC-dextran injection results suggest that approximately 30-50% of injected cells survive quantitative delivery of injected material (24 hrs post injection), then approximately 20-50% of successfully injected cells transiently expressed rsGFP 5-24 hours post injection. In general, the rsGFP-expressing cells appear to be in very good condition, with normal morphology. However, there are occasional expressing cells whose condition deteriorates with time. No significant difference in the expression frequency among $CD34^+$, $CD34^-/CD38^-$, and $CD34^+/CD38^-/Thy\text{-}1^{lo}$ populations was observed. This data provides dispositive evidence for transgene expression in primitive $CD34^+/CD38^-/Thy\text{-}1^{lo}$ cells. Further studies will be required to elucidate the gradual decline in the number of rsGFP-expressing cells. However, this pattern is not unexpected since almost all expression should be from unintegrated DNA copies—and expression should decrease as the unintegrated copies become degraded.

EXAMPLE 8
EXPRESSION AND PURIFICATION OF AAV Rep^{78}; M-MuLV INTEGRASE PROTEINS

Integration of microinjected DNA sequences is facilitated in some aspects of the invention by flanking them with specific AAV (ITR) or M-MuLV (LTR) sequences, and coinjecting them with proteins (AAV Rep^{78} or M-MuLV Integrase, respectively), which by interacting with these sequences, will enhance and/or facilitate their integration. Bacterially expressed and purified protein has been used in these studies. A fusion protein consisting of the maltose binding protein (MBP) fused to AAV Rep^{78} has been expressed in bacteria. It has been purified to approximately 90-95% homogeneity by double passage over an amylose column (MBP-Rep^{78} 120 kD, total protein obtained ~1 mg; Figure 4). The MBP-Rep^{78} expression construct used is described in Batchu *et al.* (1995). The MBP-Rep^{78} fusion protein, purified in the identical manner, was reported herein to exhibit *in vitro* binding to the AAV ITRs, endonuclease activity, and helicase activity [Batchu *et al.*, 1995]. The

present investigators purified material exhibits binding to the AAV ITR sequences. Additional experiments confirm that the binding of MBP-Rep^{78} to AAV ITR sequences is sequence specific (i.e. minimal binding to control, irrelevant DNA sequences) and results in the expected endonucleolytic cleavage of the ITR hairpin structure. These data demonstrate that the purified MBP-Rep^{78} protein has *in vitro* activity consistent with that of Rep^{78} alone. It is therefore expected that it will be active in microinjected cells. Should the fused MBP sequences interfere with integration activity, they will be removed from Rep^{78} by cleavage with factor Xa (a factor Xa cleavage site in present at the site of fusion between MBP and Rep^{78}). A similar construct for expression of an MBP-Integrase fusion protein has been generated in the present inventor's laboratory (the M-MuLV Integrase sequences described in Johnson *et al.*, (1993). Sequencing of the expression construct has confirmed the appropriate in-frame fusion. The fusion protein has already been over-expressed in bacteria and has already been purified to ~70-80% homogeneity by single passage over amylose (Fig. 5; MBP-Integrase 88 kD). Similar GST- and histidine-Integrase fusions have previously been shown to function in *in vitro* integration reactions. Again if MBP interferes with Integrase activity, MBP will be released by factor Xa cleavage. Alternatively, the GST-Integrase fusion protein will be expressed and purified [Dotan *et al.*, 1995]).

EXAMPLE 9
LINEARIZED LTR-DNA AND ITR-DNA CONSTRUCTS

For evaluation of integration strategies in hematopoietic stem cells, constructs will be employed that are capable of expressing both the red shifted Green Fluorescent Protein (rsGFP) reporter gene and the human MGMT gene. The rsGFP reporter gene in microinjection has been employed by the present investigators. It permits rapid assessment of transgene expression, without the need for additional antibody labeling or enzymatic assays. In addition, it may be employed for in vitro selection of successfully transduced stem cells prior to transplantation into humans. The human MGMT transgene will be employed because its expression in stem cells is sufficient to protect them from the toxic effects of specific alkylating agents (e.g. BCNU; [Maze *et al.*, 1996]), allowing rapid in vitro selection of gene-modified stem cells. We believe that inclusion of MGMT as a selectable marker gene may eventually enable *in vitro* or *in vivo* enrichment of stem cells co-transduced with a therapeutic gene (e.g. glucocerebrosidase). rsGFP and human MGMT will be employed

from the rsGFP-MGMT family of constructs (Fig. 6; all of these constructs have already been generated). The phosphoglycerate kinase (pgk) promoter, highly active in immature and mature hematopoietic cells, as well as in fibroblasts, will be used to drive expression of both rsGFP and MGMT genes.

There are two potential strategies for stable in vitro transduction of quiescent hematopoietic stem cells. The first is to facilitate the integration event directly in the quiescent cell ex vivo (denoted 'integration-quiescent'). The second is to deliver the DNA (perhaps together with integration enzymes) to the quiescent cell and establish a stable transduction intermediate, which can later be integrated when the cell is returned to its in vivo environment and undergoes a self-renewing cell division (denoted 'integration-quiescent/cycling'). There is evidence (AAV vectors [Russell *et al.*, 1994] and HIV-1 based vectors [Naldini *et al.*, 1996]), for the existence of such transduction intermediates in quiescent cells, capable of subsequent integration when cells go into cycle.

A plasmid backbone containing the AAV ITRs has been completed, and the rsGFP-MGMT sequences inserted between the Pac I and Asc I sites to generate prsGFP-MGMT/ITR. Linearized ITR-flanked rsGFP-MGMT sequences will obtained by digestion with Fse I and Sfi I. Circularized rsGFP-MGMT/ITR sequences, absent the plasmid backbone, will be generated as diagramed in Figure 9. The Sfi I site has been converted in prsGFP-MGMT/ITR to create a second Fse I site. The construct will be linearized and backbone plasmid sequences removed via Fse I digestion, religated to circularize at the Fse I site. and circular molecules purified on an agarose gel prior to microinjection.

Co-delivery of Integrase with LTR-flanked transgenes and co-delivery of Rep^{78} with ITR-flanked transgenes are expected to increase the rate of integration. Examining both linear and circular ITR-containing constructs will demonstrate which substrate Rep^{78} utilizes for wild-type AAV integration.

The joint delivery of naked DNA (i.e., DNA not within a delivery vehicle such as liposome) and Integrase or Rep^{78} to cell nuclei has not previously been attempted, and as part of the present invention provides an improved approach to genetically modifying important cell types.

EXAMPLE 10
LTR/INTEGRASE AND ITR/Rep^{78}-MEDIATED INTEGRATION

A characteristic of retroviruses (both lentiviruses (e.g. HIV-1) and type C retroviruses (e.g. M-MuLV)) and wild-type AAV is the ability to incorporate gene sequences, with high efficiency, into the chromosomal DNA of the target cell. Provided that the multiplicity of infection is sufficiently high, a significant fraction, if not all, of the target cells may acquire an integrated proviral copy. The only retroviral LTR sequences required for in vitro integration reactions are the extreme 10-12 bp LTR termini [Goff, 1992; Goodarzi *et al.*, 1995; Reicin *et al.*, 1995; LaFemina *et al.*, 1991]. The retroviral Integrase enzyme interacts with these sequences to facilitate integration in vitro and in vivo [Goff, 1992; Goodarzi *et al.*, 1995; Reicin *et al.*, 1995; LaFemina *et al.*, 1991]. There appears to be no site specificity for integration of retroviral vectors, although there is a preference for integration into the chromatin of transcriptionally active genes [Vijaya *et al.*, 1986; Withers-Ward *et al.*, 1994] and a preference with respect to nucleosomal conformation [Pruss *et al.*, 1994]. Wild-type AAV is unique in its preferential integration into a specific region of the human genome. AAV Rep^{78} is required for chromosome 19 specific integration, since rep$^-$ AAV vectors do not have the same pattern of site-specific integration as rep$^+$ AAV vectors [Muzyczka, 1992]. The Rep^{78} protein is believed to play several essential roles in chromosome 19 specific AAV integration -- mediating the formation of a complex between a 12 nucleotide sequence within the ITRs and a similar sequence on chromosome 19 (the AAVS1 region) prior to integration, and also introducing a strand-specific break within AAVS1 [Linden *et al.*, 1996]. It was recently reported that co-transfection of cells with an ITR-flanked gene (lacZ) together with a Rep^{78} expression construct yielded a 10 fold increase in the number of stable lacZ-expressing cells. In addition, the majority of integrants were Chromosome 19 specific and constructs up to 35 kb in size could be integrated - provided they contained at least a single ITR [Natsoulis *et al.*, 1995]. These results strongly indicate that simple delivery of ITR-flanked sequences together with Rep^{78} protein will also facilitate integration - likely to occur in a site-specific, or at least site-preferred, manner. Providing the purified Integrase or Rep^{78} protein together with the delivered DNA, possibly as a preformed DNA/ protein complex, may have advantages over producing the integration proteins in cells from an expression construct. Already delivering an ΔLTR/Integrase or ITR/Rep^{78} DNA/protein complex will

eliminate the need for the expressed proteins to translocate from the cytoplasm to the nucleus, and then to find and form complexes with the ΔLTR or ITR sequences.

HIV-1 is capable of delivering proviral DNA to nuclei of quiescent cells and stably transducing quiescent macrophages [Wenberg *et al.*, 1991; Lewis *et al.*, 1992]. Although it has not yet been conclusively demonstrated that HIV-1 based vectors can integrate in G_0 cells, they are capable of establishing a stable transduction intermediate in quiescent Rat 208F fibroblasts ('integration-quiescent/cycling') [Naldini *et al.*, 1996]. Cells recruited from quiescence even 8 days after original infection had stable transduction frequencies equal to 50% of those infected while in a cycling state. We have chosen to initially examine HIV-1 Integrase (rather than M-MuLV Integrase) will be examined for its ability to facilitate integration. HIV-1 is known to be capable of the 'full-site' integration events in vitro [Goodarzi *et al.*, 1995] and to stably transduce quiescent cells. Whether M-MuLV Integrase is also capable of accomplishing integration in quiescent cells is unknown, since pre-integration complexes of wild-type M-MuLV and M-MuLV vectors are incapable of transport into the nuclei of quiescent cells [Miller *et al.*, 1990]. However, previous studies have indicated that the actual integration of M-MuLV sequences need not occur during mitosis [Roe *et al.*, 1993].

High frequencies of integration are also observed for cells transduced with rep⁻ AAV vectors [Muzyczka, 1992]. Although the precise structure of the AAV vector DNA prior to integration is unknown, it is likely that it either exists as a linear duplex flanked by ITRs or, as is postulated to be the case for wild-type AAV, is circularized in the nucleus prior to integration [Linden *et al.*, 1996]. AAV vector DNA integrates into cellular DNA as one to several tandem copies joined to chromosomal DNA through the ITR termini [Flotte and Carter, 1995]. When linked to other transgene sequences, the ITRs alone are sufficient to confer increased rates of integration [Philip *et al.*, 1994]. There is controversy regarding the ability of rep⁻ AAV vectors to directly transduce non-cycling cells [Podsakoff *et al.*, 1994; Russell *et al.*, 1994]. Although AAV-vectors prefer cycling cells for transduction, they either directly integrate at low frequency in non-cycling cells ('integration-quiescent') [Podsakoff *et al.*, 1994] or exist as single stranded episomes in non-cycling cells and subsequently integrate as double stranded DNA when the cells go through S-phase ('integration-quiescent/cycling') [Russell *et al.*, 1994].

The ability of Integrase to facilitate integration of linearized ΔLTR-flanked rsGFP-MGMT sequences. The rsGFP-MGMT sequences will be flanked by the most terminal 20 bp HIV-1 LTR sequences to generate rsGFP-MGMT/ΔLTR. This linear construct will be generated by PCR employing primers 35 bases in length - 15 bases corresponding to the extreme rsGFP-MGMT sequences and 20 bases identical to HIV-1 LTR termini. Cells will be microinjected with either PCR-generated linear molecules of rsGFP-MGMT/ΔLTR pre-incubated with Integrase, or with PCR-generated linear molecules of rsGFP-MGMT/ΔLTR alone. Since 20-30 kb sequences can be generated by PCR amplification using optimized protocols, this approach should be generally applicable to larger transgene constructs. For evaluation of the ITR/Rep^{78} strategy, cells will be microinjected either with linearized rsGFP-MGMT/ITR pre-incubated with Rep^{78}, or linearized rsGFP-MGMT/ITR alone. Circular double-stranded proviral DNA may be the relevant substrate for normal AAV integration. This will be evaluated by employing cir-rsGFP-MGMT/ITR. In order to maximize the possibility that the integration enzymes locate and bind to their target LTR or ITR sequences, DNA and proteins will be incubated together prior to microinjection into a cell. Optimization of integration frequency will likely require varying the following experimental parameters: a) # of DNA molecules delivered per cell, b) # of Integrase or Rep^{78} molecules per DNA molecule, and c) conditions for preloading of DNA with Integrase or Rep^{78}. In addition, it will be determined if the rsGFP-MGMT/ITR integration events were in the Chromosome 19 region preferentially used by wild-type AAV -- by performing PCR with primers that span the potential junction between the AAV genome and Chromosome 19 sequences.

EXAMPLE 11
STABLE TRANSDUCTION WITH CO-DELIVERY OF INTEGRASE$_{HIV-1}$ AND ΔLTR$_{HIV-1}$ FLANKED TRANSGENE CONSTRUCTS

The present example demonstrates the utility of the present invention for increased frequency of stable transduction with co-delivery of Integrase$_{HIV-1}$ together with transgene constructs flanked by ΔLTR$_{HIV-1}$ sequences. The ability of Integrase$_{HIV-1}$ to facilitate integration of linearized ΔLTR$_{HIV-1}$-flanked pgk-tk sequences is also further identified. The pgk-tk sequences were flanked by the most terminal 20 bp HIV-1 LTR U3/U5 sequences to generate pgk-tk/ΔLTR$_{HIV-1}$ (Figure 7). This linear construct was generated by PCR

employing primers 35-36 bases in length, with 15-16 bases corresponding to the extreme pgk-tk sequences and 20 bases identical to LTR_{HIV-1} U3/U5 termini. Rat-2tk(-) cells were microinjected with either PCR-generated linear molecules of pgk-tk/ΔLTR_{HIV-1} pre-incubated with $Integrase_{HIV-1}$, or with PCR-generated linear molecules of pgk-tk/ΔLTR_{HIV-1} alone. Since 20-30 kb sequences can be generated by PCR amplification using optimized protocols, this approach should be generally applicable to larger transgene constructs. The $HIV\text{-}1_{NL4-3}$ Integrase enzyme was utilized. This integrase enzyme has been modified at two amino acids to make it more soluble, without affecting its in vitro integration ability. This general technique is known to those of skill in the art, and is described in Jenkins *et al.*, 1996. The expression construct pINSD.His.Sol may be used to express and purify additional enzyme, as needed.

Rat-2(tk-) cells were grown in Dulbecco's Modified Eagles Medium (DMEM) containing 10% Fetal Bovine Serum (FBS), 100 units/ml penicillin, 100 microgram/ml streptomycin, and 2 mM glutamine. On the day prior to injection, cells were plated at 20,000 cells per 35 mm dishes that previously had 0.4 mm rectangular grids etched into the tissue culture surface. DNA or DNA + protein were delivered into the nuclei of cells using borosilicate microinjection needles having a tip outer diameter of approximately 0.3 – 0.45 micron. Solutions of DNA alone contained approximately 42 ng/microliter of pgk-tk/ΔLTR_{HIV-1} (23 nM) in 1x microinjection buffer (50 mM Hepes [pH 7.2], 100 mM KCl, 5 mM NaH_2PO_4) containing 10 mM $MgCl_2$. Injection of 5-10 femtoliters is estimated to deliver approximately 70–140 DNA molecules per cell. Solutions of DNA and protein contained the same concentration of DNA together with 17 ng/microliter $Integrase_{HIV-1}$ (445 nM). This value was chosen to yield approximately 20 molecules of injected protein per molecule of injected DNA. The DNA solution was passed through a 0.1 micron filer to remove any aggregates including crystals believed to be $MgPO_4$.

Typically, 25-30 cells were injected per 35 mm dish, with only one cell injected per square of the gridded plate. This was done to facilitate scoring of stably transduced colonies after selection in media containing HAT (0.1 mM sodium hypoxanthine, 0.4 micro M aminopterin, 16 micro M thymidine). The day following injection, the media was removed and media containing HAT was added. HAT medium was changed every 3-5 days. 5-7 days post injection, dishes were scanned under the microscope, and the position of potential colonies growing out of stably transduced cells was marked on the bottom surface of

dish. Colonies consisted of 50-200 cells which continued to show proliferation of cells. The colonies were checked by scanning the dishes twice per week for 3 weeks. Only stable colonies (i.e., present at 3 weeks postinjection) were analyzed in the present studies.

The present studies were performed with pgk-tk/ΔLTR_{HIV-1}, with or without $Integrase_{HIV-1}$. The results are presented in Table 1. These data demonstrate that the frequency of stable transductants for pgk-tk/ΔLTR_{HIV-1} co-delivered with $Integrase_{HIV-1}$ was 4.5% vs. 1.9% for pgk-tk/ΔLTR_{HIV-1} alone. This 2.4 fold increase in stable transduction frequency was shown to be significant ($p<.05$) in a paired Student t test (2 tailed). Even further optimization may be appreciated by varying, for example, 1) the relative ratio of Integrase molecules to DNA molecules, 2) the composition of the microinjection buffer, 3) the conditions (e.g. temperature) for pre-incubating the DNA together with Integrase prior to microinjection. Even greater increases in stable transduction efficiency may thus be achieved employing the techniques of the present invention.

Table 1
HIV-1 ΔLTR/HIV-1 Integrase Strategy

Pgk-tk/ΔLTR HIV-1	Pgk-tk/ΔLTR HIV-1 + Integrase HIV-1
2/56 (3.6%)	1/47 (2.1%)
3/59 (5.1%)	5/62 (8.1%)
1/50 (2%)	3/50 (6%)
0/50 (0%)	2/50 (4%)
0/50 (0%)	2/50 (4%)
0/50 (0%	1/50 (2%)
6/315 (1.9%)	14/309 (4.5%) 2.4-fold improvement

Statistical analysis of the data in Table 1 demonstrates a statistically significant improvement in the number of cells in which stable DNA integration was obtained. This analysis revealed a statistically significant improvement (paired Student t test (2-tailed), $p<.05$)

EXAMPLE 12
STABLE TRANSDUCTION WITH CO-DELIVERY OF REP^{78} AND AAV ITR FLANKED TRANSGENE CONSTRUCTS

The present example is provided to demonstrate the use of the present invention for use in conjunction with proteins. By way of example, the protein used in the present study was an enzyme, Rep^{78}. Those of ordinary skill in the art will appreciate that many other proteins may be used given the present disclosure.

The present example demonstrates the utility of the present invention for providing increased frequency of stable transduction with co-delivery of Rep^{78} together with transgene constructs flanked by AAV ITR sequences. For evaluation of the ITR/Rep^{78} strategy, cells were microinjected either with linearized pgk-tk/ITR co-delivered with Rep^{78}, or linearized pgk-tk/ITR alone. Linearized ITR-flanked pgk-tk sequences were obtained by digestion with Fse I and Sfi I, and then gel purified to eliminate bacterial plasmid sequences. The purified MBP-Rep^{78} protein described in Figure 4 was used. MBP-Rep^{78} has been previously observed by the inventors to be active in *in vitro* ITR binding and endonuclease reactions. These studies were performed as described in Example 11 except that the DNA solution contained pgk-tk/ITR at a concentration of 50 ng/microliter (23 nM), the DNA/protein solution contained pgk-tk/ITR at 50 ng/microliter and MBP-Rep^{78} at 56 ng/microliter (475 nM), and the $MgCl_2$ concentration was 2 mM. These values were chosen to yield approximately 20 molecules of injected protein per molecule of injected DNA. Although the exact volume delivered per cell was not known, injection of 5-10 femtoliters would deliver approximately 70–140 DNA molecules per cell.

The present results are provided in Table 2. A 2.3-fold increase in stable transduction frequency was achieved when Rep^{78} was included together with pgk-tk/ITR. For these studies, the MBP-Rep^{78} protein was added to the DNA solution immediately prior to loading of solution into the microinjection needle. Comparison with an initial experiment (not shown) in which the DNA/protein solution was not prepared immediately prior to microinjection, but rather was maintained for several hours before microinjection, suggests that the timing of addition of protein to DNA may be important.

Table 2

AAV ITR/Rep^{78} Strategy

pgk-tk/ITR	pgk-tk/ITR + Rep^{78}
3/72 (4.2%)	5/80 (6.3%)
4/76 (5.3%)	8/66 (12.1%)
2/76 (2.6%)	7/72 (8.7%)
9/224 (4.0%)	20/218 (9.2%)
	2.3-fold improvement

EXAMPLE 13

CIRCULARIZED CONSTRUCTS FOR NUCLEIC ACID INTEGRATION

The present example demonstrates the utility of the present invention for the generation of covalently closed circles for facilitating integration. Since circular double-stranded proviral DNA may be the relevant substrate for normal AAV integration, and possibly for retroviral integration, it may be useful to first circularize the transgene constructs. One such circularized construct is shown for cir-rsGFP-MGMT/ITR in Figure 9. A plasmid backbone containing the AAV ITRs has been constructed, and the rsGFP-MGMT sequences inserted between the Pac I and Asc I sites to generate prsGFP-MGMT/ITR. Linearized ITR-flanked rsGFP-MGMT sequences have been obtained as described above by digestion with Fse I and Sfi I. The Sfi I site (the backbone plasmid sequences) in prs GFP-MGMT/ITR was converted to create a second Fse I site. The molecule will be linearized and removed via Fse I digestion. The molecule will then be religated to circularize at the Fse I site. The circular molecules will then be purified on an agarose gel prior to microinjection.

The above strategy may also be used to facilitate retroviral Integrase-mediated integration of transgene constructs. Although the constructs shown in Figure 6 containing ΔLTR sequences (or possibly also the complete LTR sequences) are all linear, it is possible that presenting the transgene flanked by ΔLTR or LTR sequences on a circular construct

would lead to increased frequencies of integration. For example, although the substrate for retroviral provirus integration is believed to be a linear molecule flanked by LTRs, it is conceivable that the circular proviral molecules (containing either one or two LTRs) present in the nucleus of retrovirally infected cells are also potential substrates for integration. In fact, use of such circular constructs, co-delivered with retroviral Integrase protein may lead to increased efficiency of integration in comparison with the linear form. It is possible that the LTRs, or LTR terminal sequences included in such circular constructs may be either in juxtaposition to each other, or separated by some distance. By way of example, this distance may be defined by a number of nucleic acid bases. For example, the number of nucleic acid bases separating the first and the second LTR terminal sequences could be between 2 to about 200 nucleotide bases. In some embodiments of the circularized nucleic acid the LTR terminal fragments are separated by about 10 to about 20 nucleotide bases.

EXAMPLE 14 SITE-SPECIFIC TARGETING OF TRANSGENE INTEGRATION BY EMPLOYING RETROVIRAL INTEGRASE PROTEIN FUSED TO PROTEINS HAVING HIGH SPECIFICITY OF BINDING TO SPECIFIC DNA SEQUENCES.

Wild-type AAV exhibits preferential integration into a specific region of the human genome. AAV Rep^{78} is required for chromosome 19 specific integration, since rep$^-$ AAV vectors do not have the same pattern of site-specific integration as rep$^+$ AAV vectors [Muzyczka, 1992]. The Rep^{78} protein is believed to play several essential roles in chromosome 19 specific AAV integration -- mediating the formation of a complex between a 12 nucleotide sequence within the ITRs and a similar sequence on chromosome 19 (the AAVS1 region) prior to integration, and also introducing a strand-specific break within AAVS1 [Linden *et al.*, 1996]. As such, Rep^{78} may itself be described as a protein having both a binding activity for specific DNA sequences, and an integrase activity.

There appears to be no site specificity for integration of retroviral vectors, although there is a preference for integration into the chromatin of transcriptionally active genes [Vijaya *et al.*, 1986; Withers-Ward *et al.*, 1994] and a preference with respect to nucleosomal conformation [Pruss *et al.*, 1994]. However, it may ultimately be possible to target the precise integration site by employing Integrase linked to high-specificity DNA binding domains (Fig. 2) [Goulaouic and Chow, 1996; Bushman, 1994]. The DNA binding domain

could either be derived from an existing DNA binding protein, or a novel protein could be designed and synthesized to have a high affinity binding specificity for specific genomic DNA sequences. For example, it is possible that specific targeting of transgenes to the globin locus (by fusing retroviral integrase to the DNA binding domains of GATA-1 or NF-E2 erythroid specific transcription factors) would facilitate transgene expression specifically in erythroid cells. Another potential example would be targeting the integration to chromatin known to be 'open' in monocyte/macrophages may facilitate long-term expression of therapeutic genes for lysosomal storage diseases. It may be possible to increase the affinity and specificity of DNA binding proteins for specific DNA sequences by structural analysis (e.g. X-ray crystallographic and/or NMR analysis of the interaction between the DNA binding domain and the DNA target) of the protein and site directed mutagenesis. For example, mutants of the DNA binding domain of the c-myc protein having increased affinity for DNA have been described.

EXAMPLE 15 USE OF CONSTRUCTS EXPRESSING INTEGRATION ENZYMES

In some embodiments, this invention provides for the co-delivery to a cell of a first and a second DNA sequence. This first and second nucleic acid sequence may be created as two separate constructs, one containing the transgene(s) of interest flanked by appropriate LTR or ITR sequences, and the other being an expression construct encoding a protein, particularly an enzyme capable of facilitating the incorporation of the transgene sequence into the cellular chromosomal DNA (i.e. retroviral integrase or Rep^{78}). Microinjection would be capable of co-delivering both the transgene construct and integrase-expression construct to the nucleus of cells to be genetically modified. After expression and then translation of the integrase protein in the cytoplasm, it would necessarily have to translocate to the nucleus to interact with the transgene construct to facilitate integration. It has previously been described that certain retroviral integrases localize to the nucleus after expression in cells. An expression construct for the M-MuLV Integrase under the control of the cytomegalovirus (CMV) promoter/enhancer sequences has been prepared by the present inventors. In addition, an expression construct for the AAV Rep^{78} protein under the control of the CMV promoter/enhancer sequences has also been generated.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Gene-Cell, Inc.

(ii) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Gene-Cell, Inc.
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(C) CITY: Houston
(D) STATE: Texas
(E) COUNTRY: USA
(F) ZIP: 77058
(G) TELEPHONE NUMBER: (512) 495-8400

(iii) TITLE OF INVENTION: NUCLEIC ACID CONSTRUCTS AND USES THEREOF FOR DIRECT NUCLEIC ACID INCORPORATION INTO CELLS

(iv) NUMBER OF SEQUENCES: 2

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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WHAT IS CLAIMED IS:

1. A method for increasing incorporation of a naked nucleic acid sequence of a gene of interest into a chromosomal nucleic acid of a cell comprising:

preparing a composition comprising a protein and a transgene construct, said transgene construct having a nucleic acid sequence of interest flanked by a long terminal repeat sequence or a fragment thereof, and

introducing the composition to a nucleus of the cell.

wherein stable incorporation of the nucleic acid of interest into the chromosomal nucleic acid of the cell is enhanced in the presence of the protein over incorporation of the nucleic acid of interest in the absence of the protein.

2. The method of claim 1 wherein the protein is further defined as an integrase enzyme.

3. The method of claim 1 wherein the long terminal repeat sequence is further defined as a retroviral long terminal repeat or a fragment thereof.

4. The method of claim 1 wherein the enzyme is further defined as a retroviral integrase.

5. The method of claim 1 wherein the protein is further defined as a fusion protein comprising an enzyme fused to a nucleic acid binding protein.

6. The method of claim 5 wherein the protein is further defined as comprising an enzyme fused to a DNA binding domain of GATA 1 or NFE 2 capable of binding a sequence within a globin locus.

7. The method of claim 6 wherein the enzyme is a retroviral integrase enzyme.

8. The method of claim 4 or 6 wherein the retroviral integrase enzyme is further defined as a recombinant retroviral integrase protein.

9. The method of claim 8 wherein the recombinant retroviral integrase protein is HIV-1, M-MuLV, SIV, FeLV OR EIAV.

10. The method of claim 1 wherein the cell is a mammalian cell.

11. The method of claim 10 wherein the mammalian cell is a fibroblast, hematopoietic stem cell, endothelial, mesenchymal, epithelial, or glial cell.

12. The method of claim 1 wherein the transgene construct is defined as rsGFP-MGMT/LTR.

13. The method of claim 6 wherein the nucleic acid binding protein is further defined as capable of binding a nucleic acid sequence.

14. The method of claim 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12 or 13 wherein the composition is introduced into the nucleus of a cell by microinjection.

15. The method of claim 1 wherein the transgene construct is MGMT/MDR-1.

16. A method for increasing incorporation of a nucleic acid sequence of interest into chromosomal nucleic acid of a cell comprising:

preparing a composition comprising an integration enzyme and a transgene construct having a nucleic acid sequence flanked by an adeno-associated virus inverted terminal repeat or fragment thereof; and

introducing the composition to the cell.

17. The method of claim 16 wherein the integration enzyme is Rep^{78}.

18. The method of claim 16 wherein the transgene construct is prsGFP-MGMT/ITR.

19. The method of claim 16 wherein the composition is introduced into the cell through microinjection.

20. The method of claim 16 wherein the cell is a mammalian cell.

21. A composition comprising:

a transgene construct having a nucleic acid sequence flanked by long terminal repeat or a fragment thereof; and

an enzyme capable of facilitating the integration of the nucleic acid sequence into nucleic acid of a cell.

22. The composition of claim 21 wherein the long terminal repeat sequence comprises a length of about 10 to about 20 base pairs in length.

23. The composition of claim 21 wherein the transgene construct is rsGFP-MGMT/LTR.

24. A composition comprising:

a transgene construct having a nucleic acid sequence of interest flanked by an adeno-associated virus inverted terminal repeat sequence or fragment thereof; and

an integrase protein.

25. The composition of claim 24 wherein the integrase protein is Rep^{78}.

26. The composition of claim 24 wherein the transgene construct is prsGFP-MGMT/ITR.

27. A circularized nucleic acid construct comprising:

a nucleic acid sequence of interest flanked by a first Integrase recognition sequence and a second Integrase recognition sequence (IRS)

wherein said first IRS and second IRS are in juxtaposition to each other or are between about 2 to about 20 nucleotide bases from each other.

28. The circularized nucleic acid construct of claim 27 wherein the integration recognition sequences are AAV inverted terminal repeat regions or fragments thereof.

29. The circularized nucleic acid construct of claim 27, wherein the IRS's are LTRs or fragments thereof.

30. The circularized nucleic acid construct of claim 28, wherein the LTRs are further defined as HIV-1 sequences.

31. A method for increasing incorporation of a nucleic acid sequence of interest into chromosomal nucleic acid of a cell, comprising

preparing a composition comprising a first nucleic acid sequence comprising a transgene construct containing a nucleic acid sequence of interest flanked by a terminal fragment of a long terminal repeat sequence; and a second nucleic acid sequence encoding a protein capable of enhancing the incorporation of the sequence of interest into the chromosomal nucleic acid of the cell.

32. The method of claim 31 wherein the second nucleic acid sequence encodes an integration enzyme.

33. The method of claim 32 wherein the long terminal repeat sequence of the transgene construct is further defined as a retroviral long terminal repeat sequence.

34. The method of claim 33 wherein the cell is a mammalian cell.

35. The method of claim 31 wherein the first nucleic acid sequence and the second nucleic acid sequence are not co-extensive with one another.

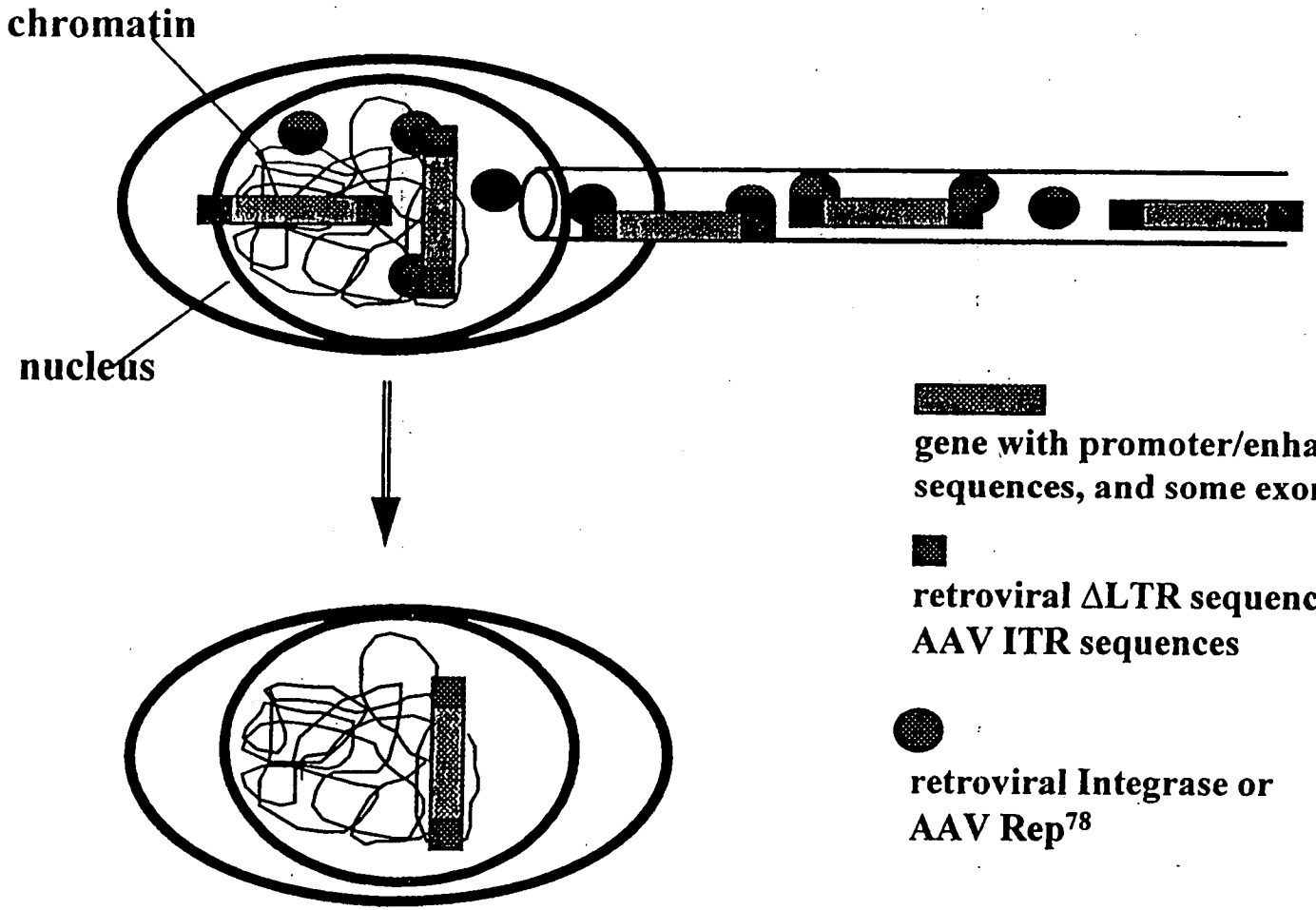

chromatin
nucleus
gene with promoter/enhancer, LCR sequences, and some exon/intron structure
retroviral ΔLTR sequences or AAV ITR sequences
retroviral Integrase or AAV Rep78

Fig. 1

Possible Strategies for Integration

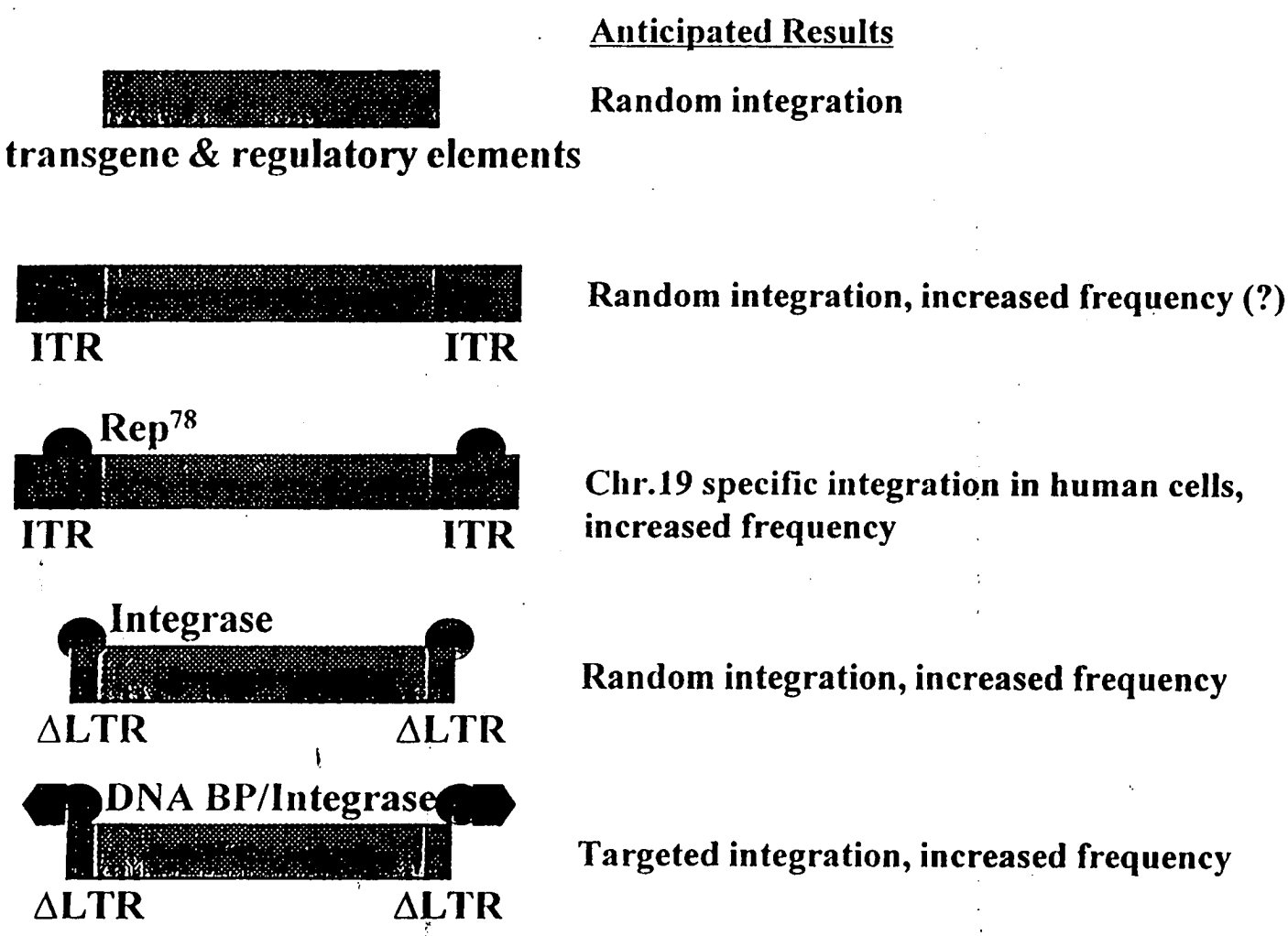


Fig. 2

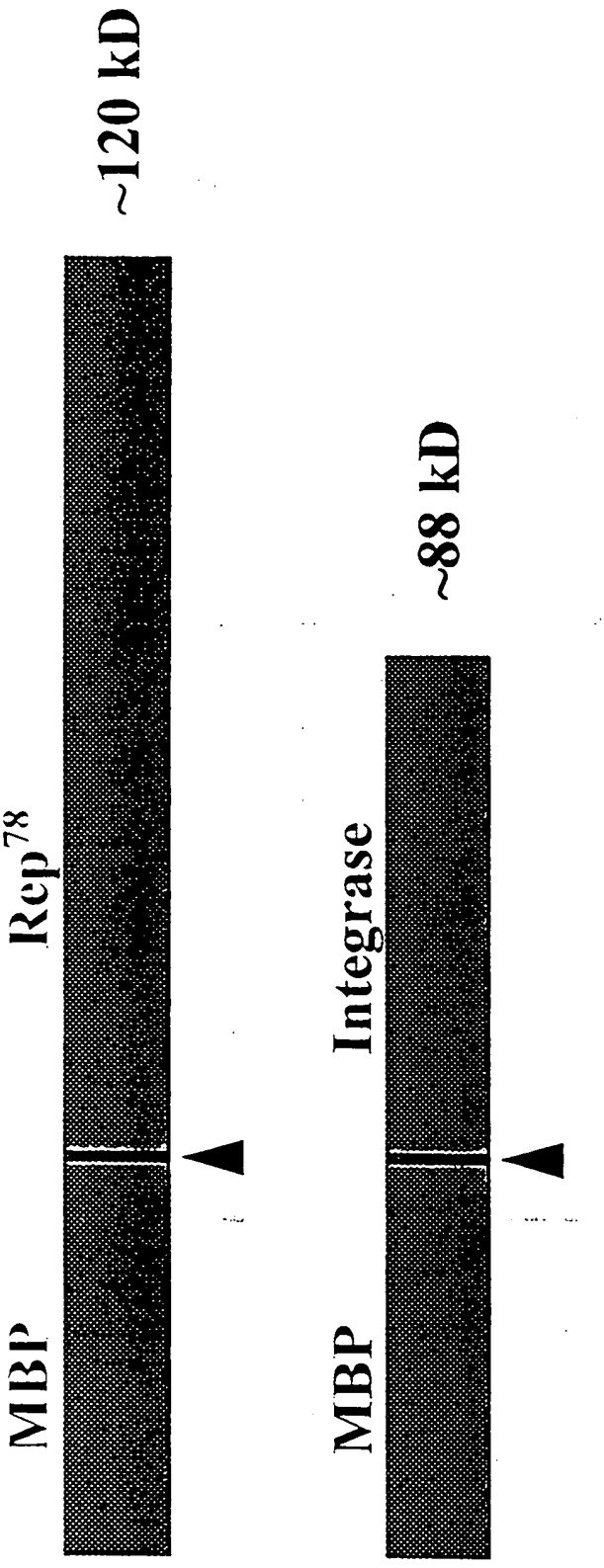


Fig. 3

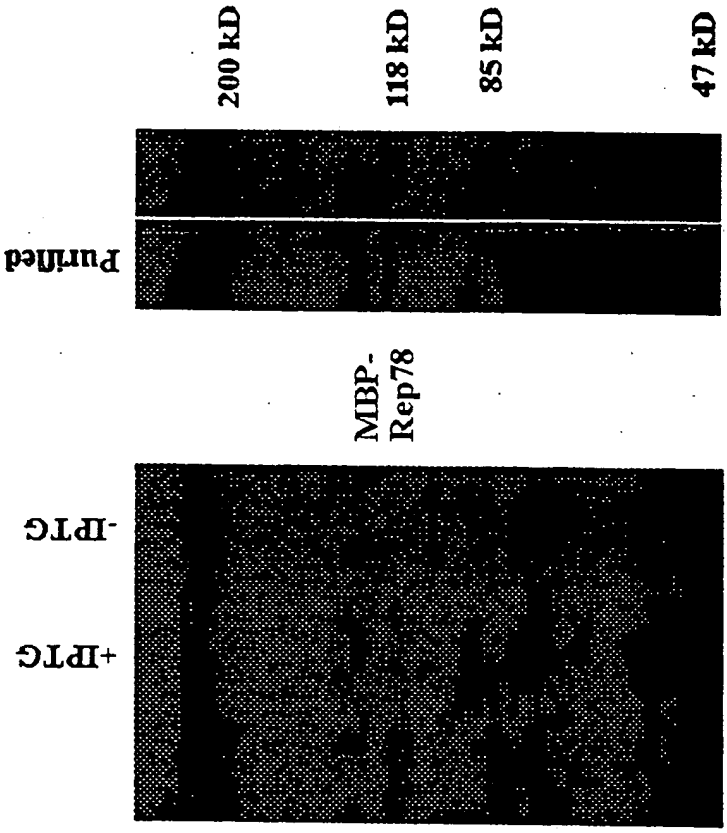


Fig. 4B

Fig. 4A

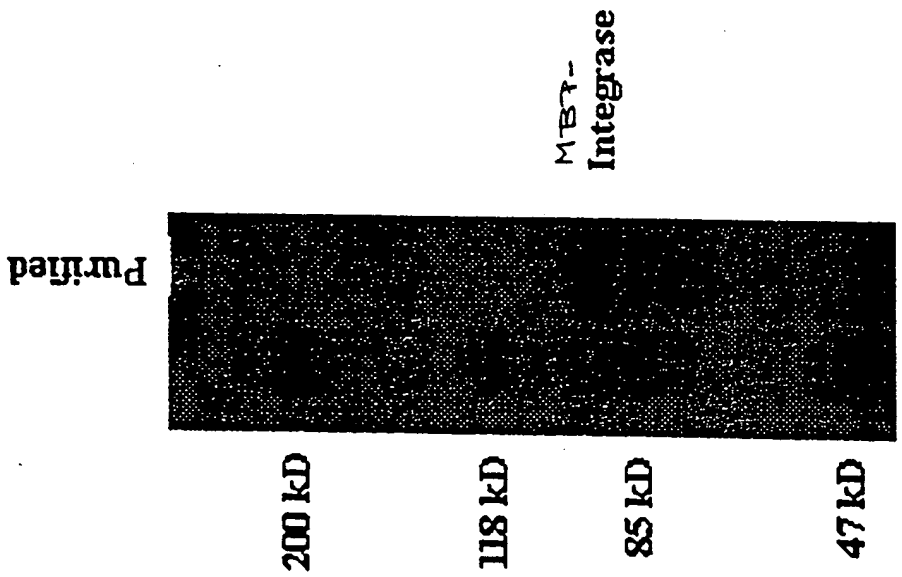
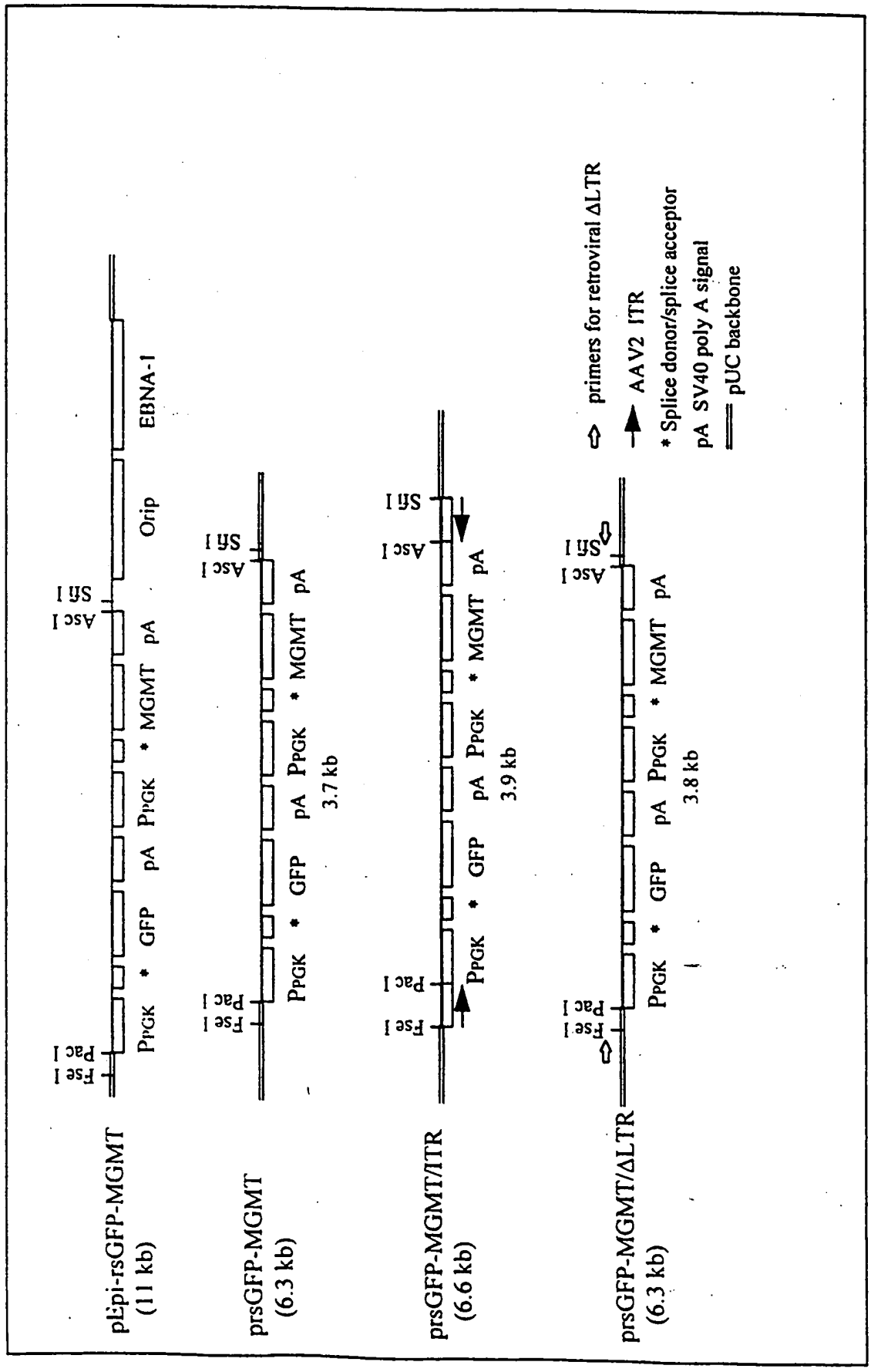


Fig. 5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Pac I
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Sfi I
Orip
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Pac I
PPGK * GFP pA PPGK * MGMT pA
Asc I
Sfi I
3.7 kb
prsGFP-MGMT/ITR
(6.6 kb)
Fse I
Pac I
PPGK * GFP pA PPGK * MGMT pA
Asc I
Sfi I
3.9 kb
prsGFP-MGMT/ΔLTR
(6.3 kb)
Fse I
Pac I
PPGK * GFP pA PPGK * MGMT pA
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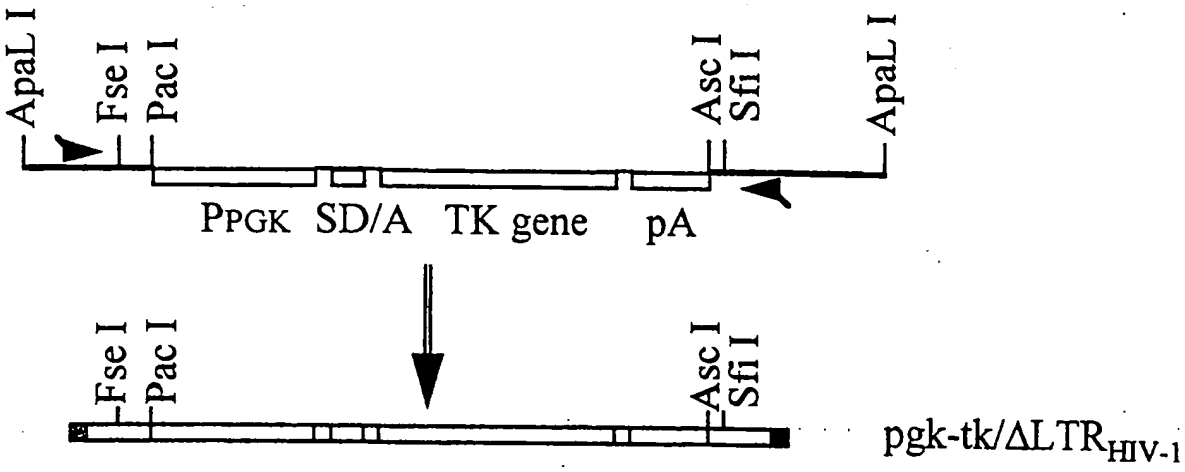
Sfi I
3.8 kb
primers for retroviral ΔLTR
AAV2 ITR
* Splice donor/splice acceptor
pA SV40 poly A signal
pUC backbone

Fig. 6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Pac I
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Fse I
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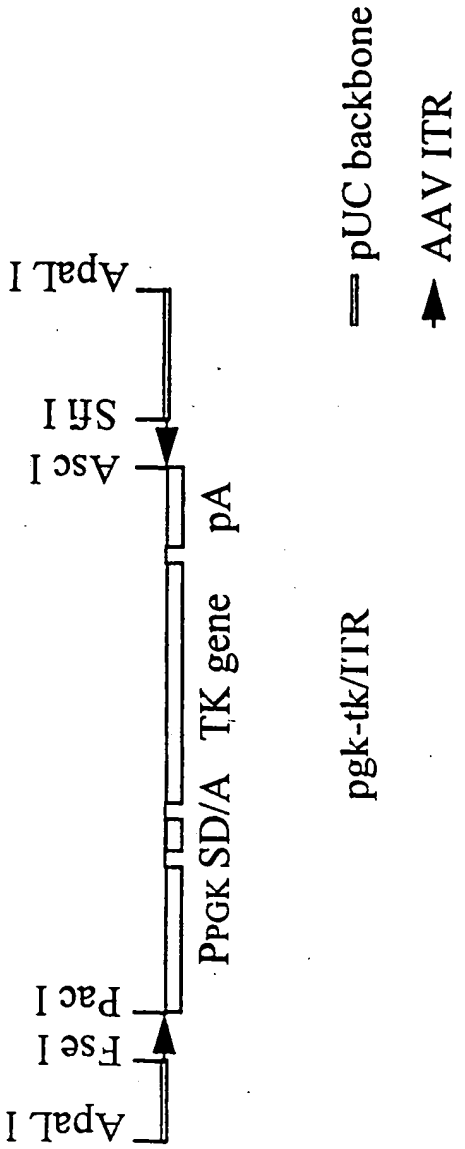
Asc I
Sfi I
pgk-tk/ΔLTR$_{HIV-1}$

Fig. 7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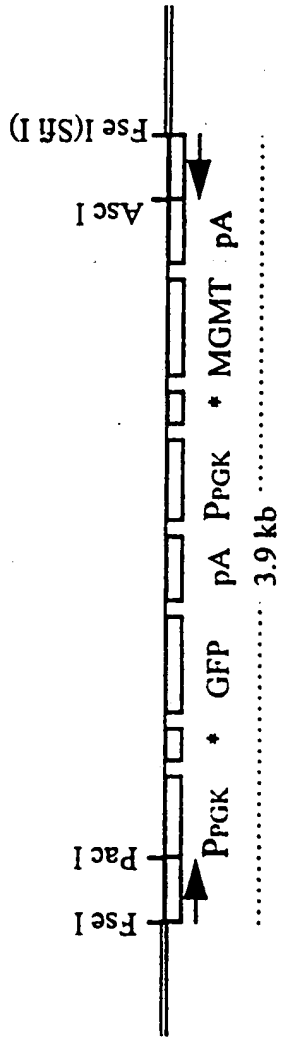
pgk-tk/ITR
pUC backbone
AAV ITR

Fig. 8



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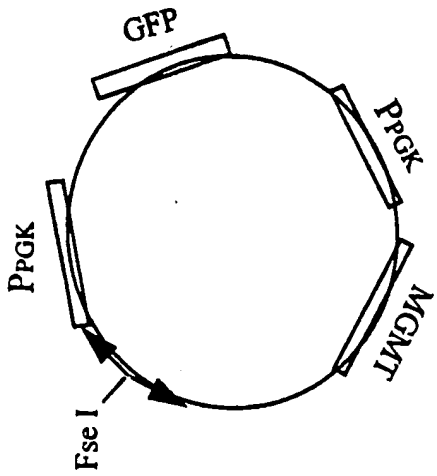

cir-rsGFP-MGMT/ITR
(3.9 kb)

Fig. 9

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/24236

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 15/11, 15/63, 15/64

US CL :435/172.3, 320.1; 536/23.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/172.3, 320.1; 536/23.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ---- Y	SHOJI-TANAKA et al. Gene Transfer Using Purified Retroviral Integrase. Biochemical and Biophysical Research Communications. 30 September 1994. Vol. 203. No. 3. pages 1756-1764, especially pages 1757-1758.	1-4, 8, 10-12, 15, 21-23, 27, 29-35 -------- 5-7, 9, 13-14, 16-20, 24-26, 28
Y	WO 95/32225 A1 (THE SALK INSTITUTE FOR BIOLOGICAL STUDIES) 30 November 1995, see entire document, especially pages 4-5.	5-9, 13-14

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 26 FEBRUARY 1998	Date of mailing of the international search report 20 MAR 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer TERRY A. MCKELVEY Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, DIALOG
search terms: stable, incorporat?, long terminal repeat?, transfect?, transform?, integrase, retrovir?, DNA binding protein, GATA1, NFE2, globin, rsGFP-MGMT/LTR, MGMT/MDR-1, AAV, adeno-associated virus, rep78